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Potential Development of Resistance to Food Antimicrobials by Foodborne Pathogens

Lilia Milagros Santiago Santiago
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Lilia Milagros Santiago Santiago entitled "Potential Development of Resistance to Food Antimicrobials by Foodborne Pathogens." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

P. M. Davidson, Major Professor

We have read this dissertation and recommend its acceptance:

David A. Golden, Alan G. Matthew, Ann F. Draughon

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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POTENTIAL DEVELOPMENT OF RESISTANCE TO FOOD ANTIMICROBIALS BY FOODBORNE PATHOGENS

A Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Lilia Milagros Santiago Santiago
May, 2006

Dedication

I would like to dedicate this dissertation to my parents Victor and Milagros, my brother Victor Jose, my sister Yanira and especially in loving memory of my dearest grandparents Damian Santiago, Josefa Rivera, Tito Santiago and Josefa Diaz.

Acknowledgments

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Abstract

The potential for development of resistance of four strains of *Listeria monocytogenes* (101, 108, 310 and Scott A) and *Salmonella* Typhimurium DT 104 (2380, 2576, 2582, 2486) to food antimicrobials sodium benzoate (SB), potassium sorbate (PS), sodium diacetate (SD) and sodium lactate (SL) at pH 6.0 was studied. Cells were not subjected to any pre-stress condition. An agar dilution assay was performed to determine susceptibility and adaptation to different and increasing antimicrobial concentrations. A microbroth dilution assay was used to determine tolerance development after two consecutives exposures to same antimicrobial concentrations, and one further exposure to a step higher concentration. A growth curve was made with four strains (101, 108, 2380, 2486) to compare growth of adapted vs. non-adapted (parent) strains at same high concentrations in different medium environments. In the agar dilution assay, both microorganisms demonstrated increased resistance after growth in the presence of the food antimicrobials up to 0.5% PS, 4.0% SL and 1.0% SD. For SB, *Listeria* cells adapted up to 0.4% (except strain 108) and *Salmonella* cells to up to 0.5%. Using a microbroth dilution assay, all strains showed less susceptibility to low levels (0.1-0.3%) of PS and BS. Results indicated that 4.0% SL had essentially no effect on any cells growth. For all strains there was no change in absorbance (OD_{630}) at >1.0% SD. Adapted strains grown in TSB with and without dextrose resulted in higher log CFU/ml compared to non-adapted strains when exposed to previous and higher antimicrobial concentrations. Both microorganisms showed potential for resistance to SL and to lower concentrations of BS and PS at certain pH.

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PART ONE

PART ONE: REVIEW OF THE LITERATURE

I. Introduction

In recent years, the focus on safer foods and longer shelflife has led to more frequent use of regulatory approved food antimicrobials and chemical sanitizers in order to inhibit and control growth of foodborne pathogens such as *Listeria monocytogenes*, *Salmonella,* and *Escherichia coli* O157:H7. Despite some success in using these compounds for such control, questions have been raised concerning the potential for development of resistance to these compounds. If these compounds are to be utilized as a major part of a pathogen control system, information on the potential for resistance development must be investigated. Currently, there is little data on the possible emergence of foodborne pathogens resistant to regulatory approved food antimicrobials and sanitizers (Davidson and Harrison, 2002).

The potential for target pathogenic microorganisms to develop resistance to food antimicrobials and sanitizers has been linked to the increasing incidence of microorganisms exhibiting resistance to antibiotics used for therapeutic purposes in human and animal medicine. Today we are encountering multi-resistant microorganisms in clinical and farm settings that are difficult to combat with currently available antibiotics. These multiple resistances have been mainly attributed to the proliferation of resistance genes and to the ease of dissemination of resistant strains between humans and animals especially via food of animal origin or fecal contamination (van den Bogaard & Stobberringh, 1999).

Antibiotic resistant bacteria entering the food-processing environment may pose a contamination risk to processed food. Antibiotics used at sub-therapeutic levels to

promote rapid growth at the farm-level and to improve feed conversion into meat and milk, can leave residues that then could contribute to the development of resistance to other antimicrobial compounds (Brady and Katz, 1992; Brady et al., 1993).

Recent studies indicate that the direct use of certain food antimicrobials and sanitizers may impose a selective pressure and contribute to the emergence of resistant microorganism in food environments (Russell, 2000). In the food-processing environment, bacteria are exposed to multiple stresses (e.g., heat, antimicrobials compounds), which are mainly used to preserve quality, increase shelflife and improve safety. Sublethal preservation stresses may result in cells that are less susceptible to subsequent stresses (Ravishankar and Juneja, 2003). Under such conditions, bacteria present in the plant environment survive and may be able to adapt to even harsher treatments (Ravishankar and Juneja, 2003).

Resistant pathogens surviving traditional regulatory-approved food antimicrobials and sanitizers represent a threat to the food industry and consumers. It is crucial to continue investigations of the potential for resistance development and monitor the proper and adequate use of these antimicrobial compounds in order to preserve the safety of the food supply of the future.

II. Antibiotic Resistance and its Relationship to Food Processing

A. History of Development of Antibiotic Resistance by Bacteria

The search for antibiotics began in the late 1800s with the growing acceptance of the germ theory of disease (Bass et al., 2001). At that time, scientists began the search for drugs that would kill pathogenic microorganisms. The goal of such research was to find drugs, or so-called "magic bullets" that would destroy microorganisms without being toxic to the person taking the drug (Wainwright, 1990).

In 1888, the German scientist E. de Freudenreich found that the blue pigment released in culture by the bacterium *Bacillus pyocyaneus* arrested the growth of other bacteria in the cell culture (Bass et al., 2001). After many experiments, he showed that pyocyanase, the product isolated from *B. pyocyaneus*, could kill many pathogenic bacteria. The major problem with pyocyanase was that it proved to be toxic and unstable so it could not be developed into an effective drug (Bass et al., 2001).

The major discovery concerning the development of antibiotics occurred in 1928 when the British scientist Alexander Fleming discovered penicillin. After returning from a weekend vacation, Fleming discovered that some old Petri dishes had some colonies of *Staphylococcus* that had lysed. He observed that bacterial cell lysis occurred in an area adjacent to a contaminant mold growing on the plate and hypothesized that a product of the mold had caused the cell lysis (Bass et al., 2001). Later, Fleming demonstrated that the mold produced a low molecular weight substance that lysed bacteria such as *Staphylococcus aureus*. The substance was named penicillin after the *Penicillium* mold

that had produced it. By 1946 the drug had become widespread for clinical use in the United States (Bass et al., 2001).

The widespread clinical use of penicillin for treatment of all kinds of ailments, led Fleming to warn that misuse of penicillin could lead to selection of resistant forms of bacteria (Levy, 1992). Fleming proved the existence of resistant mutants by varying the dosage and conditions upon which he added the antibiotic to bacterial cultures. Fleming reported that resistance to penicillin could be conferred in two ways: either through the strengthening of the bacterial cell wall, which the drug destroyed, or through the selection of bacteria expressing mutant proteins capable of degrading penicillin (Levy, 1992). Antibiotic resistance was identified in bacterial pathogens very soon after the introduction of penicillin into clinical practice.

One of the major problems with misuse of penicillin was that the drug was available to the public without prescription until the mid 1950s. People used penicillin as cure-all and even used it to treat non-bacterial diseases. In 1946 a hospital reported that 14% of the strains of *Staphylococcus* isolated from sick patients were penicillin resistant (Levy, 1992). By the end of the decade, the same hospital reported that resistance had been conferred to 59% of the strains of *Staphylococcus* studied (Levy, 1992).

Development of resistance to antibiotics has been shown to be primarily attributed to significant variations in the microbial population and extensive genetic modifications such as the acquisition of resistance genes. These transferable resistance genes arise from acquisition of "foreign" DNA elements by conjugation, transduction or transformation

(Mazel and Davies, 1999). Resistance transfer may occur via clonal spread of bacteria transfer of resistance genes populations (van den Boogaard and Stobberingh, 1999).

The frequent use of antibiotics may result in sublethal concentrations in the environment, which, in turn, may lead to selective pressure for retention of these resistance genes among microorganisms (van den Boogaard and Stobberingh, 1999). Considerable progress has been made to understand more fully the resistance mechanisms and responses involved among the most resistant types of bacteria. Some major resistance mechanisms include activation of multi-drug efflux systems and the synthesis of degradative enzymes. Antibiotic resistance is attributed to resistance genes, which often code for proteins that have been present in bacteria for an extremely long time. These proteins function by protecting the bacterial ribosome or coding for proteins that serve as efflux pumps, both of which maintain the integrity of the bacterial cell (Singer, 2003).

B. Impact of Resistant Bacteria in the Food Industry

1. Antibiotic Resistance in Bacteria Associated with Foods

The significance of antibiotic resistant bacteria and its association with foodborne pathogens is matter of continuing scientific debate. The spread of antibiotic resistant pathogenic bacteria that infect humans through the food chain has been a controversial subject for almost 40 years (Threfall, 1992). Concerns about the use of antibiotics in food animals have focused on food safety because resistant bacteria, including zoonotic foodborne pathogens, can be transmitted to humans through the consumption or handling

of foods of animal origin (Tollefson and Karp, 2004). The use of antibiotics in food animals has been a human health concern since the 1970s when FDA first called for restrictions on antibiotics used in animal feed (Bren, 2001). In recent years, attention has been focused on the emergence of therapeutic-antibiotic resistant strains among the most common foodborne pathogens. These include emerging resistant phenotypes among foodborne pathogens such as *Escherichia coli*, *Salmonella*, *Listeria monocytogenes* and *Campylobacter* spp. The resistance of these bacterial strains to a variety of antibiotics has become a major health concern since it has diminished the effectiveness of treatment and led to the use of less safe, ineffective alternatives (Kiessling et al., 2002; Morell, 1997). While most foodborne illnesses are not treated with antibiotics, if a particular foodborne pathogen present in a food and is antibiotic resistant, a person taking that antibiotic and consuming the contaminated food is more susceptible to that pathogen. Additionally, if a person becomes ill via an antibiotic resistant foodborne pathogen, treatment with antibiotics will be ineffective.

One of the major problems with the use of antibiotics is misuse, causing the possible widespread distribution of resistant bacterial isolates in other environments like foods and farm animals (Aarestrup, 1999). In human medicine antibiotics are mainly used to treat bacterial infections or bacterial diseases. But in food-producing animals, antibiotics are used for three major purposes namely: therapy (for the treatment of an identified bacterial infection), prophylaxis (prevention of bacterial infections in animals at risk), or as feed additives to enhance weight gain (van den Boogaard and Stobberingh, 1999). It is estimated that in the US, 23,000,000 kg of antibiotics are produced each year, of which

more than 40% are for animal use and 80% of those are used as growth promoters (Willis, 2000).

Modern food animal production practices provide conditions favorable to the selection and spread of resistance bacteria. Food-producing animals are typically raised in large groups with high animal densities. Although individual animals may be treated with antibiotics, it is common practice to treat entire groups of animals when a few animals become ill (Tollefson and Karp, 2004). The more an antibiotic is used, the more likely resistant populations are to develop among pathogens and commensal bacteria of animals in an exposed population (Philips et al., 2004) Commensal bacteria, which constitute a large reservoir of resistance genes, may also be transferred from food animals to humans. Many of the antibiotics administered to food animals are either identical to or related to drugs that are also used to treat foodborne diseases in humans. This is a problematic since resistance genes frequently encode resistance not just to a particular antibiotic but also to an entire class of antimicrobials, and resistance to several different antibiotics may emerge when only one antimicrobial drug is used (Tollefson and Karp, 2004). Indeed, multi-drug resistance among foodborne pathogens and other bacteria is becoming more and more common. An important example is the appearance in the 1960s and 1970s of the multi-drug resistant *Salmonella* of bovine origin that caused many infections in humans (Threlfall et al., 1978). During the last decade the same single multi-resistant clone, better known as *Salmonella* Typhimurium Definite Phage Type (DT) 104, has spread world-wide and has become the most common cause for human salmonellosis in several countries (Aarestrup, 1999). The microorganism is resistant to at least the

following seven antibiotics: ampicillin, chloramphenicol, gentamycin, kanamycin, streptomycin, tetracycline and sulphonamides (Threfall et al., 1999).

Antibiotic resistant bacteria have also been associated with probiotics and starter cultures in foods. Probiotics and starter cultures have been known to serve as hosts for antibiotic resistance genes that can be transferred to foodborne pathogenic bacteria. Danielsen and Wind (2003) found that most *Lactobacillus* seemed to be resistant to antibiotics like penicillin, oxacillin, cefoxitin, cephalothin and vancomycin. The levels of resistance of *Lactobacillus* varied depending on the strain and the mechanisms of action.

In human food handling, control of the emergence of bacterial resistance is based on hygienic measures, such as prevention of cross contamination (van den Boogaard and Stobberingh, 1999). The primary means of preventing transmission of antibiotic resistance through the food chain is the same as for controlling foodborne pathogens i.e., use of proper food handling and food preparation techniques (Singer, 2003). The continued availability of effective antibiotics will ultimately depend upon the responsible use of these products. The key point is to look for strategies to control use and minimize spread of resistance bacteria among populations both in humans and animals.

Many efforts are being made to reduce the selection of resistant bacteria and help to preserve these valuable antimicrobial drugs for both humans and animals. Organizations like the World Health Organization (WHO) has made the following recommendations to reduce use and misuse of antibiotics in food animals to protect public health: (1) obligatory prescriptions for all antimicrobials used for disease control in food animals, (2) pre-approval evaluation of food animal antimicrobials to assess their risk of causing

resistance, (3) identification of emerging health problems through resistance monitoring so that timely corrective actions may be taken to protect human health, and (4) development of prudent use guidelines for veterinarians and producers (Tollefson and Karp, 2004).

III. Mechanisms of Resistance to Food Antimicrobials and Antibiotics

The ability to adapt and change under stress conditions and various environments is a consequence of the development of microbial resistance to antimicrobial agents. There is more information about mechanisms of resistance to therapeutic antibiotics than about food antimicrobials. This is because antibiotics have specific target sites in a microbial cell and the development of resistance to these compounds is a result of changes in these sites (Davidson and Harrison, 2002). Mechanisms of resistance to food antimicrobials are not fully understood and more difficult to predict. In both cases, mechanisms of resistance may vary depending on the physiological status of the organism, the type and concentration of antimicrobial and the physicochemical characteristics of the external environment.

Bacterial resistance to any type of antimicrobial compound may be mediated by multiple mechanisms and/or resistance determinants in the bacteria cell. Some cells will grow and survive after antimicrobial exposure because they may possess a degree of natural resistance, or may acquire it later through mutation or genetic exchange (Bower and Daeschel, 1999). Two types of parameters may describe resistance responses of microorganisms to food antimicrobials and therapeutic antibiotics: intrinsic or innate resistance and extrinsic or acquired resistance.

A. Intrinsic Parameters

Intrinsic resistance, also known as innate resistance, is a chromosomally controlled property associated with the microorganism. Since food antimicrobials and sanitizers

have a generally broad spectrum, it is believed that resistance is most likely due to unspecified reduced uptake controlled primarily by innate characteristics within the organisms (Russell 1991; Russell et al., 1997). Most bacteria are resistant to the action of toxic compounds because of inherent mechanisms that protect cells (Fernandes, 2003). The primary mechanisms associated with intrinsic or innate resistance include microbial cell impermeability, inactivation of toxic compounds via microbial enzymes and efflux pumps (Davidson and Harrison, 2003).

1. Altered Permeability

Resistance to antimicrobial agents like antibiotics and food antimicrobials can be due to adaptation in the cell envelope. The outer layers of a bacterial cell that limit the cell uptake of antibacterial agents impart cellular impermeability. Antimicrobials compounds must penetrate the cell envelope and attain a high enough concentration at the target site to exert antibacterial action (Cloete, 2003). Inhibition will also depend on the affinity of these antimicrobial agents to the outermost layers and how easily these agents can penetrate the cell. Many food antimicrobials require a particular hydrophile-lipophile balance for optimal activity. Hydrophilic properties allow the compound to migrate to the water phase where microbial growth occurs. Lipophilic characteristics appear to be required to allow the antimicrobial to be attracted to and react with the membrane of the microorganisms (Davidson and Branen, 2005). The role of the cell wall as a barrier to the penetration of toxic molecules differs considerably between Gram positive and Gram negative bacteria (Figure 1.0).

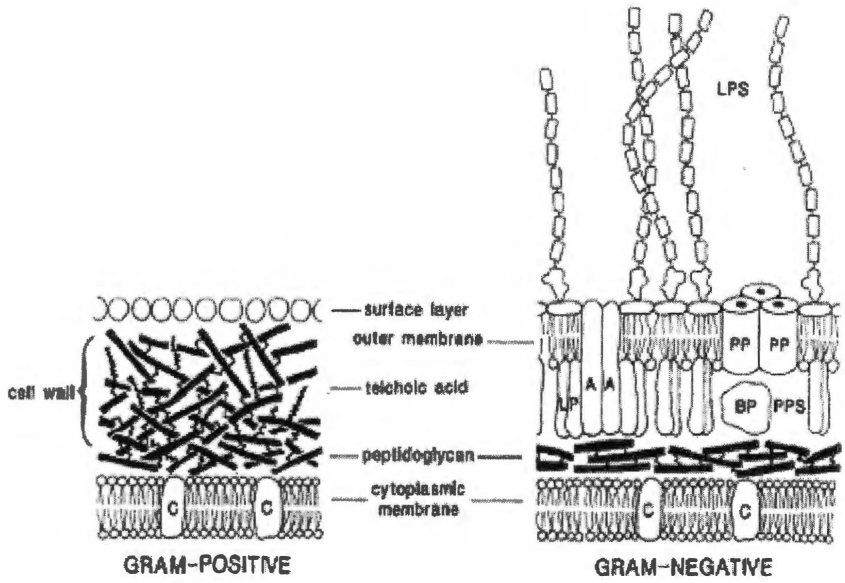

Figure 1.0 Cell envelope of Gram positive and Gram negative bacteria. PP, porins; C, cytoplasmic embedded protein; BP, binding protein; PPS, periplasmic space; A, other membrane proteins; LPS, lipoprotein. (Adapted from Sikkema et al., 2002)

The role of the cell wall as a barrier to the penetration of toxic molecules differs considerably between Gram positive and Gram negative bacteria. Gram negative bacteria are protected from antimicrobial agents by negatively charged lipopolysaccharides in their outer membrane that limit the entry of hydrophobic antimicrobial compounds into the cell (Hogan and Kolter, 2002). Small hydrophilic molecules can cross the membrane through non-specific porins while larger more hydrophobic compounds can only enter the cell through specialized porins (Hogan and Kolter, 2002). This exclusion barrier is one of the factors that accounts for the greater resistance of Gram negative bacteria to antimicrobials compounds compared with Gram positive bacteria. Gram positive bacteria

have permeable cell walls with an open, hydrophilic structure, which retains the cell shape when isolated and purified (Lambert, 2000). The major component of Gram positive walls is peptidoglycan, which accounts for 50% of the weight of the wall (Koch, 2000). Linear anionic polymers, termed teichoic acids are covalently linked to the peptidoglycan giving the wall a net negative charge. Teichoic acids are linear polymers of repeating units of ribitol or glycerol units linked by phospodiesters (Koch, 2000). Gram positive bacteria have no specific receptor molecules or permeases to assist or block bactericide penetration. Therefore, substances of high molecular weight can readily traverse the wall. This provides a plausible explanation for the sensitivity of organisms such as *Staphylococcus* and of vegetative *Bacillus* spp., to antimicrobial agents including quaternary compounds and chlorhexidine (Russell, 1995; McDonnell and Russell, 1999). Small molecular weight antimicrobials are not excluded on the basis of their size so substances like phenols and alcohols can also penetrate the wall with ease. Therefore, resistance of Gram positive to antimicrobial compounds is mostly related to mechanisms involving destruction or inactivation of toxic compounds, changes in the target site, or active efflux of the chemicals out of the cell (Russell, 1998).

The absence of an outer membrane is associated with the lower tolerance to antimicrobial agents by Gram positive compared to Gram negative bacteria. One factor that does alter susceptibility is the thickness and degree of cross-linking of the peptidoglycan (Russell, 1995).

The multi-component barrier system of Gram negative bacteria regulates passage of substances into and out of the cell and is responsible for the impermeability of these

microorganisms to antimicrobial agents. The outer cell layer of Gram negative bacteria consists of an outer membrane (envelope), a thin layer of peptidoglycan and a periplasmic space situated between the outer membrane and the peptidoglycan/cytoplasmic membrane. The outer envelope is a bilayer structure composed of lipopoylsaccharides (LPS) and phospholipids, with proteins, in particular porins, embedded in that membrane (Denyer and Maillard, 2002). Modifications of these components can cause an alteration of outer membrane permeability and lead to changes in antimicrobial susceptibility (Tattawasart et. al., 2000). The lipopolysaccharides (LPS) layers in the outer membrane of Gram negative bacteria consist of three covalently linked regions: the lipid A, the central core polysaccharide and the outer O-polysaccharide side chain. Lipid A is a phosphorylated glucosamine disaccharide unit to which a number of fatty acids are attached and its deletion results in increased susceptibility to hydrophobic antimicrobials. The core of polysaccharide is a complex oligosaccharide that is linked to the lipid A. The O-side chain is composed of many repeating units of oligosaccharides and has great diversity between and within bacterial species (Denyer and Maillard, 2002). LPS are non-covalently cross-linked and held in position at the outer membrane surface by divalent cations. These are essential for the structural integrity and strength of the outer membrane (Nikaido and Vaara, 1985).

All the components of the cell envelope play a role in the barrier mechanisms, except peptidoglycan, which is spongy and permeable (Cloete, 2003). Gram negative bacterial LPS prevent ready access of hydrophobic molecules to phospholipids and hence to the cell interior (McDonnell and Russell, 1999). Low-molecular weight hydrophilic

molecules readily pass via the porins into Gram negative cells, but hydrophobic molecules must diffuse across the outer membrane bilayer.

The major permeability barrier in any membrane is the lipid bilayer and its barrier property is inversely correlated to its fluidity (Nikaido, 1994). The cytoplasmic membrane is composed of lipids and proteins and is described as a fluid mosaic in which globular proteins are embedded in the phospholipids matrix or bilayer (Russell, 2005). The initial stages of inhibitory organic acids are binding and penetration into lipid bilayer (Ramos et al., 2002). Microorganisms may alter their susceptibility to antimicrobials by modifying membrane fluidity. Fluidity is changed by altering the phospholipids or fatty acid composition (i.e. chain length or saturation) through mechanisms similar to those observed in response to physical and chemical stresses imposed by the environment (Ramos et al., 2002). At the molecular level, this includes changes in temperatures or presence of lipophylic agents (Ingram, 1976). Ingram (1977) reported on changes in the fatty acid and phospholipids components of *E. coli* cells grown in the presence of sublethal concentrations (i.e. 26mM sodium sorbate, 20 mM sodium benzoate) of a variety of organic acids and food additives. These changes in fatty acid profiles were consistent with the hypothesis that cells adapted their membrane lipids to compensate for the presence of these compounds in the environment (Tomlins et al., 1982). Juneja and Davidson (1992) reported that the sensitivity of *Listeria monocytogenes* Scott A and ATCC 19114 were altered when bacterial membrane lipid composition was modified by growth in the presence of added fatty acids. The study indicated that *L. monocytogenes* Scott A with increased C14:0 or C18:0 fatty acids had significantly higher MIC for

methyl paraben (1637 μg/ml and 1900 μg/ml respectively) compared to control cells (1412 μg/ml). The same pattern was observed for propyl paraben, TBHQ and sodium chloride.

Another well known intrinsic mechanism of resistance to antimicrobials is formation of biofilms. Biofilms are defined as bacterial populations adhering to a surface or to each other in aggregates by a matrix of polysaccharides (Paulsen, 1999). The glycocalyx matrix in a biofilm is a polyanionic polymer that acts as an exchange resin and adsorbs or neutralizes biocides protecting the bacterial cell (Russell and Chopra, 1990; Brown et al., 1995). Microorganisms in biofilms are 100-1000 times less susceptible than equivalent populations of planktonic cultures to several types of antimicrobial agents such as biocides, including iodine and iodophors, glutaraldehyde, and chlorhexidine (Gilbert et al., 2002; Morton et al., 1998). It is well known that microorganisms become more resistant in a biofilm since the matrix forms a protective barrier against the effect of antimicrobial agents (Ravishankar and Juneja, 2003). It has been known that nutrient limitation and reduced growth rates can increase the resistance of bacteria to antimicrobials (Brown and Williams, 1985).

2. Enzymatic Degradation

Many bacteria produce enzymes that chemically degrade antimicrobial agents rendering them ineffective. Here the antimicrobial is either degraded or modified by enzymatic activity before it can reach the target site and damages the bacterial cell (Hugo and Foster, 1964). The enzymatic degradation of antibiotics such as β-lactams is a well-

known phenomenon and an important mechanism of resistance (Russell, 2005). Strains of both Gram negative and Gram positive bacteria have been found to produce enzymes capable of inactivating antibiotics such as penicillin and cephalosporins (Bower and Daeschel, 1999). Others enzymes can convert a bactericide to a non-toxic form that prevents them from exerting damage to the cell. Wright (2003) reported that resistance to aminoglycoside antibiotics is primarily the result of expression of enzymes that covalently modify the antibiotics, either by acetylation or phosphorylation. This modification resulted in interference with binding to the target 16S rRNA in the ribosome blocking the bactericidal effect of the antibiotic. Cellular targets can be altered by mutation or enzymatic modification in such a way that the affinity of the antimicrobial agent for the target is reduced (Putman et al., 2000).

3. Efflux Pumps

Certain bacteria can often become resistant to antimicrobials through a mechanism known as efflux pumps. Efflux pumps were first described for tetracyclines by McMurry et al. (1980). The primary function of the efflux pumps is to defend the cell against naturally occurring environmental toxicants (Miller and Sulavick, 1996). An efflux of pump is essentially a channel that actively exports antimicrobials and other toxic compounds out of the cell. The antimicrobial enters the bacterium through a porin and this is pumped back out of the bacterium by the efflux pump (Figure 2.0). By actively pumping out antimicrobials, the efflux pumps prevent the intracellular accumulation

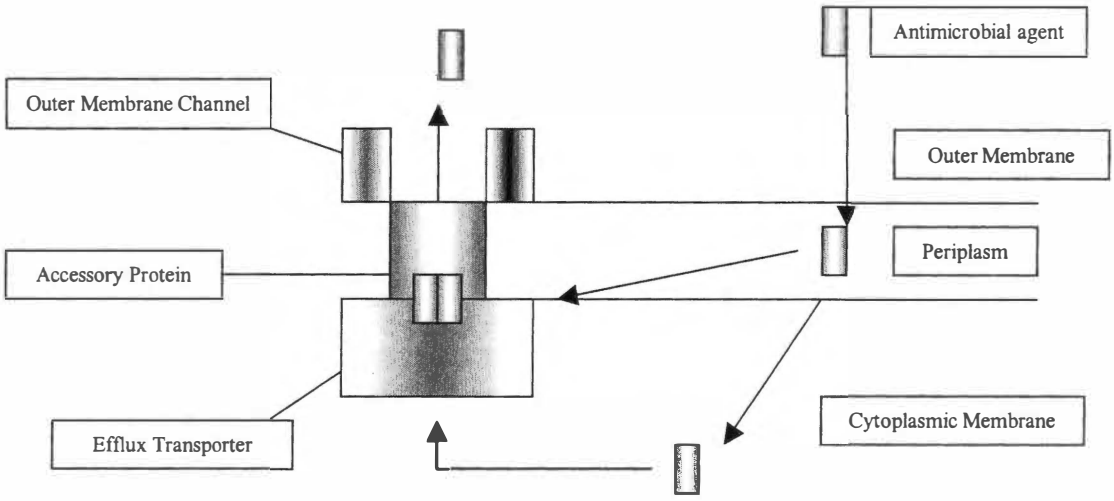

Figure 2.0 Structure and mechanism of action of efflux pumps to expel antimicrobial agents via porins outer membrane channels.
(Adapted from Nikaido, 1994)

necessary for lethal activity (Bower and Daeschel, 1999). Efflux pumps have been observed in many foodborne pathogens including *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* (Borges et al., 2003; Galimand et al., 2003; Fernandes et al., 2003; Levy, 2002; Poole, 2002; Ryan et al., 2001; Mata et al., 2000; Zgurskaya and Nikaido, 2000; Nikaido, 1998, 1999, 2001; Saier et al., 1998; Paulsen et al., 1996; Russell and Day, 1996).

The majority of efflux transporters share a common three-component organization: a transporter located in the inner membrane (IM), an outer membrane (OM) channels which functions with the IM, and a periplasmic accessory protein (Zgurskaya and Nikaido, 2000). These latter will "bridge" the cytoplasmic transporter and an outer membrane channel so that antimicrobial agents can be extruded directly into the

surrounding medium rather that into the periplasm (Nikaido, 1994; Saier, et al., 1998). Accessory proteins occur together with many efflux transporters especially in Gram negative cells.

Efflux pumps vary both in their specificity and mechanism (Hogan and Kolter, 2002). Energy-driven efflux systems can be chromosomally located or acquired by bacteria, and can either be activated by environmental signals or by mutation in a regulatory gene (Nikaido, 2001; Oullette and Kundig, 1997). Two major categories of efflux pumps exist: those dependent on ATP and those energized by a proton motive force (PMF) (Levy, 2002). Single-drug transporters include ATP-binding cassette (ABC) transporters, which use ATP as an energy source to pump the toxic compounds out of the cell (Ryan et al., 2001). For PMF, toxic compounds are removed by the efflux process that is coupled to the influx of protons (H^+) (Borges et al., 2003; Paulsen et al., 1996). In bacteria, tetracycline pumps are prototypical examples of proton motive force-dependent single polypeptide efflux pumps in bacteria (Nikaido, 1994). Protons are transported from the bacterium across the cytoplasmic membrane to the periplasmic space. As the hydrogen ions (H+) accumulate on one side of a membrane, an electrochemical gradient or potential difference is created across the membrane (Keiser, 2001). The energized state of the membrane as a result of this charge separation is called proton motive force (PMF). At low concentrations of antimicrobial, changes in cell permeability may cause depletion of the PMF because the pH gradient is depleted. This will also occur at higher concentrations to decrease the membrane potential (Montville et.al., 1995). The trans-

membrane proton motive force driven efflux pumps is seen more in bacteria than the ABC transporters that are mainly found in eukaryotes (Ryan et al., 2001).

Energy-driven efflux pumps systems may be specific for certain types of antimicrobials or have a broad spectrum like the so called "multiple drug resistance pumps" (Levy, 2002). Multiple drug resistance efflux systems are classified into the small multidrug resistance (SMR) family, drug/metabolite transporter (DMT) superfamily, major facilitator superfamily (MFS), ATP-binding cassette (ABC) family, resistance-nodulation-division (RND) family and multidrug and toxic compound extrusion (MATE) family (Poole, 2002). It has been suggested that the major energy-dependent active efflux pumps belong to RND family identified mainly in Gram negative bacteria (Ramos et al., 2002).

B. Extrinsic Parameters

Acquired resistance results from genetic changes in the microbial cell through direct mutation of the chromosome acquisition of plasmids, transposons, or other genetic elements (McDonnell and Russell, 1999; Russell 1991; 2003). Acquired resistance occurs primarily among bacteria and toward antibiotics. This is because antibiotics used for therapeutic purposes generally have specific target sites in microbial cells and therefore have greater selective pressure for development of acquired resistance (Davidson and Harrison, 2002). Resistance to food antimicrobials and sanitizers is likely non-specific and primarily by innate factors (Russell et al., 1997). However, genetic modifications to increase resistance cannot be ruled out.

1. Genetic Transfer

Bacteria can acquire genes that permit the survival of the cell to exposure to different antimicrobial compounds. Resistance genes may be acquired by pathogenic bacteria from a pool of genes in other microbial genera, and by a variety of genetic exchange and transfer mechanisms that include conjugation, transformation and transduction (George, 1996). Horizontal gene transfer of antimicrobial resistance genes is mainly achieved through transformation and conjugation. It is believed that conjugation is the major mechanism by which Gram negative bacteria exchange DNA, including antimicrobial resistance genes (Woo et. al., 2003). Conjugation involves direct cell-to-cell contact for transfer of extra-chromosomal DNA (Barbosa and Levy, 2000). Conjugation is mediated by a plasmid that replicates independently of the chromosome. Many plasmids carry genes that confer resistance to antimicrobials. Sidhu et al., (2001) reported that strains of *Staphylococcus* harbored both sanitizer (QAC) and antibiotic resistance (penicillin) determinants on the same plasmid associated with DNA mobile elements. Resistance by *Staphylococcus* to QAC by the *qac A/B* gene system results from efflux but is mediated by the selection of *qac A* genes on multi-resistance plasmids (Russell, 2001). The multidrug resistance ability of some microorganisms has been attributed to "R" or resistance plasmids. Resistance genes in plasmids are mostly inserted by site-specific recombination within transposable elements or integrons cassettes. Integrons are DNA elements capable of mobilizing individual gene cassettes into bacterial chromosomes by site-specific recombination.

Transformation is the process where genes are transferred from one bacterium to another as "naked" DNA. When cells die and break apart, DNA can be released into the surroundings environment. Other bacteria in close proximity can scavenge this free-floating DNA, and incorporate it into their own DNA. This DNA may contain advantageous genes, such as antimicrobial resistant genes, that benefit the recipient cell (Barbosa and Levy, 2000). It is believed that transformation is the major mechanism by which Gram positive bacteria uptake DNA encoding antimicrobial resistance genes from the environment (Woo et al., 2003).

In transduction, bacterial DNA is transferred from one bacterium to another by a bacteriophage. When a phage infects a bacterium, bacterial DNA may inadvertently be incorporated into new phage DNA (Barbosa and Levy, 2000). Upon bacterial death and lysis, new phage goes on to infect other bacteria which may brings genes from the previously infected bacterium.

2. Mutations

Mutation rate is an estimate of the rate (per generation) of mutation per nucleotide, per locus, or eventually, for the whole genome, and involves favorable, unfavorable or neutral mutations (Martinez and Baquero, 2000). In the case of antibiotic resistance, the mutation rate is frequently defined as the in vitro frequency at which detectable mutants arise in a bacterial population in the presence of a given concentration of antimicrobial (Martinez and Baquero, 2000). A major reason for bacterial resistance to antibiotics is associated with mutational changes in cellular target sites (Russell, 2005). In recent years,

it has been demonstrated that the mutation process in bacterial populations is not a static event. Mutation rates can change for a given antibiotic depending on its concentration during selection. Physiological conditions, such as the availability of a particular carbon source or general bacterial stress, also may regulate mutation rate in bacteria (Foster, 1993; Hughes and Anderson, 1997; LeCler et al., 1996; Shapiro, 1997; Taddei et al., 1997).

The probability that a mutation will give an antibiotic resistance phenotype (mutability) influences the mutation rate. Mutability will depend on the structure and the number of genes in which mutations can produce a selectable phenotype (Martinez and Baquero, 2000). A variety of genes can be involved in antibiotic resistance either because there are several different target, access, or protection pathways for the antibiotic in the bacterial cell or because each the pathway requires expression of several genes. If several genes are required for the access of the antibiotic to its target, mutations in each of the genes will produce an antibiotic resistance phenotype (Martinez and Baquero, 2000). Once a mutation causes an antibiotic resistance phenotype, the bacteria carrying the mutated allele must compete with the wild-type bacterial population. Dominance will depend on the relative fitness defined as the efficiency of multiplication of the mutant cell compared with that of the wild-type ancestor population (Elena and Lenski, 1997). An important aspect in mutations is competition with other organisms that will depend on the environment in which bacteria will grow. Structured and compartmentalized environments (like surfaces) allow bacteria to occupy different niches and thus not necessarily compete with each other (Martinez and Baquero, 2000). Under these

circumstances, all possible alleles in the population capable of surviving the selective pressure will grow. The growth on structured habitats increases the variability of bacterial populations and accelerates their evolution in response to environmental inputs.

C. Bacterial Stress Responses

Gould (1989) reported that vegetative bacterial cells exposed to environmental stresses adapted to the stresses in a variety of ways to maintain a homeostatic condition. These adaptations included activation and expression of new groups of genes. Rowbury (1998) suggested that secreted extracellular components could be involved in inducing resistance responses by effectively warning bacteria of impending stress. When some microorganisms are exposed to certain types of stress, i.e., antimicrobials, low pH or extreme temperatures, they may activate two types of cellular responses: a general stress response or a specific stress response. The first is: general stress response. General stress responses are a large group of genes that facilitate growth and survival under different stress conditions (i.e., osmotic shock, pH or thermal stress). The second cellular response is the specific stress response that occurs due to specific stimuli such as heat shock. It is under the control of a specific regulator. Two of the most common genetic regulatory factors are the *mar* operon from the multidrug resistance (*Mdr*) system and Sigma (σ) factors. Both regulatory factors are frequently involved in enhanced stress resistance responses.

1. *Mar* operon

Multidrug resistance (*Mdr*) is a term used to describe intrinsic genetic mechanisms for resistance that constitute part of the normal genome of cells. *Mdr* in bacteria is generally attributed to the acquisition of multiple transposons and plasmids bearing genetic determinants for different mechanisms of resistance (Alekshun and Levy, 1999). *Mdr* genes are activated by induction or mutation and activation of which is the result of exposure to stress by xenobiotics (i.e., synthetic chemicals believed to be resistant to environmental degradation) in natural and clinical environments (George, 1996). The only phenotypic mechanism identified for *Mdr* in bacteria is drug efflux by membrane transporters, even though many of these putative transporters remain to be identified (Poole, 1994; Levy, 1992; Nikaido, 1994; Ma et al., 1994). The ability of bacterial cells to efflux xenobiotics is a complex phenomenon that can involve a combination of reduced influx and increased efflux within the cell envelope (Nikaido, 1994; Ma et al., 1994). A major *Mdr* system in Gram positive bacteria is a system that has a single gene that encodes a membrane transporter for the efflux of many unrelated drugs (George, 1996). In Gram negative bacteria, in addition to single gene-encoded multidrug exporters, there are also operons or regulons that encode repressors and transcriptional activators and genes at the other loci that are regulated by activators (George, 1996).

The most studied regulon in the *Mdr* systems is the chromosomally-controlled mar-operon (*marRAB*). This operon generates multiple antibiotic resistance (*mar*) phenotypes and is a member of the multidrug resistance systems found in Gram negative bacteria such as *E.coli*, *Pseudomonas aeruginosa* and *Salmonella* Typhimurium (George and

Levy, 1983; Kunonga et al., 2000; Levy, 2002; Piddock et al., 2000). Deletion of the *marRAB* operon results in increased susceptibility to multiple antibiotics, a variety of oxidative stress agents, and organic solvents (Alkeshun and Levy, 1999).

Genetic organization of the mar locus includes regulatory proteins encoded by *marRAB*. This includes *marR*, *marA* and *mar B*. *marR* (144 amino acids), a repressor of other proteins to allow activation of *marA* and negatively controls *marRAB* expression by binding to another protein called *marO*. *MarA* (127 amino acids residues) that activates transcription of *marRAB* by binding to *marO* and other operons of the *Mar* regulon. *MarB* (72 amino acids) is a protein essential for resistance but still has an unknown function (Kunonga et al., 2000). The *mar* regulon functions by direct inactivation of *marR* or indirectly by inducing compounds (i.e., tetracycline, chloramphenicol, benzoate, etc.) that enter through outer membrane porins or by diffusion (Kunonga et al., 2000). Genes with known functions that respond to *marA* and that are thus within the *mar* regulon include *acrAB* genes (a stress-induced efflux system), which then represses the synthesis of *ompF* (outer membrane protein F), the point of entry for some antibiotics, altering the expression of other membrane proteins (Alkeshun and Levy, 1999). The *mar* locus is present in many members of the *Enterobacteriacea* family and is conserved at the DNA sequence level (Alkeshun and Levy, 1999). The *mar* locus and operon in *E. coli* and other members of the *Enterobacteriacea* is the generalized response locus leading to increased expression of efflux pumps (Levy, 2002). The most studied *marRAB* region is in *Salmonella enterica* serovar Typhimurium. Some of the most common substances inducing the *mar* operon include tetracycline, chloramphenicol, dinitrophenol,

menadione, paraquat, plumbagin, benzoate, and sodium salicyclate, the latter being the most potent inducer known (Randall and Woodward, 2001).

The *mar* operon may be responsible for tolerance of some microorganisms to structurally unrelated antimicrobial agents, such as antibiotics, organic solvents and biocides (Fernandes et al., 2003). Moken et al., (1997) found that mutations in *marR*, which allowed *marA* expression and activation of the multidrug efflux, pump *acrAB* to pump out a household product like pine oil or an antibiotic such as tetracycline. McMurry et al., (1998) found that overexpression of the multidrug efflux pump locus *acrAB*, or of *marA*, which encodes positive regulators of acrAB, decreased susceptibility to triclosan 2-fold. Deletion of the *acrAB* locus increased the susceptibility to triclosan approximately 10-fold. Four of five clinical *E. coli* strains that overexpressed marA also showed enhanced triclosan resistance. The *acrAB* locus was involved in the effects of triclosan upon both cell growth rate and cell lysis. Levy (2001) reported that deletion of the *acrAB* gene complex (a stress-induced efflux system) in different bacterial cells like *E. coli*, caused reduction of the growth inhibition by triclosan from 8μg/ml to 3-4μg/ml proving the importance of the gene in the resistance to the antimicrobial agent.

2. Sigma Factors and *rpoS*

Survival by bacteria under stress conditions depends on the presence of adaptive mechanisms and the ability of the organism to respond at the molecular level by through response expression of regulons and/or stimulons in order to avoid or repair damage (Bacon et al., 2003). Bacterial cells enter to a stringent respond whenever the supply of

energy or amino acids is limited (Rees et al., 1995). This occurs mainly under stress conditions such as adverse pH, osmolarity and temperature. A central regulator of stationary-phase gene expression has been identified in bacteria growing under stress conditions. The central regulator for stationary-phase gene expression is the *rpoS* gene product that is responsible for the induction of a specific subset of bacterial genes only expressed under stress conditions and modulated by alternative sigma (σ) factors (Lande and Hengge-Aronis, 1991). The primary role of the sigma (σ) factors is to bind to core RNA polymerase conferring promoter specificity (Abee and Wouters, 1999). RNA polymerase is the enzyme responsible for the transcription of DNA into RNA, which ultimately concludes in protein synthesis (Rees et al., 1995). Alternative sigma factors are encoded by genes under *rpoS* control and induced on entry into stationary phase. Levels of *rpoS* gene products are controlled through by the amount of RNA transcript being made and read (Dodd and Aldsworh, 2001). *RpoS* are regulated at the transcriptional, translation and post-translational levels of a large number of genes (i.e. osmB, membrane lipoprotein; glgS, glycogen synthesis) required for environmental stresses, including growth phase-acid dependent acid tolerance (Davidson and Harrison, 2002). The function of the sigma factor is to recognize and bind to transcriptional signals (promoter sequences) in the primary DNA sequence (Rees et al., 1995). This binding in turn promotes the correct alignment of the RNA polymerase. Once a transcriptional unit (i.e., gene) has been identified, transcription is initiated and the gene is expressed (Rees et al., 1995). Transcription of the *rpoS* genes occurs throughout growth but is specifically induced by weak acids, high osmotic pressure and by entry into the stationary phase

(Rees et al., 1955). The onset of stationary phase is believed to be signaled by changes in metabolism which occur in response to nutrient limitation or any reduction of growth rate induced by a variety of environmental stresses (Rees et al., 1995). The *rpoS* genes are known to be positively regulated by the starvation-specific module *ppGpp* (guanisone 31-diphosphate-51-diphosphate, guanisone tetra phosphate) accumulated as part of the stringent response (Gentry et al., 1993). Stringent response may be defined as the response that induces the synthesis of alternative sigma factors which then co-ordinates the expression of many genes important for the long-term cell survival (Rees et al., 1995).

Some of the most studied sigma factors include the alternative stress response sigma factor σ^S which is specific to the stationary phase, the alternative *sigB* encoding σ^B (sigma B) which control a transcription of genes involved in environmental stress and acid adaptation, and *rpoD* encoding σ^A which controls housekeeping genes and *Gad* (glutamate decarboxylase) genes in microorganisms such as *L. monocytogenes* and *E.coli* (Wemekamp-Kamphuis et al., 2004; Rees et al., 1995). Housekeeping genes are responsible for transcription of the majority of the proteins that are synthesized under specific stresses such as heat-shock and chemotaxis. Transcription of the *rpoS* gene could also occur throughout bacterial growth but is specifically induced by weak acids (Schellhorn and Stones, 1992), high osmotic pressure (Lange and Hengge-Aronis, 1994) and by entry into stationary phase (Rees et al., 1995).

The regulon σ^B is a secondary unit of RNA polymerase that is the most known to govern a large stress response regulon in *Bacillus subtilis, L. monocytogenes* and *S.*

aureus (Becker et al., 1998; Kazmierczark et al., 2003). In *L. monocytogenes*, the σ^B regulon is related to physiological and chemical changes and responses that may serve as primary osmosensor in this organism. *L. monocytogenes* also responds to different environmental stresses by activating two important systems: membrane bound histidine kinase (HK) and a transcriptional response regulator (RR) (Kallipollitis and Ingmer, 2001). The response regulators are activated to ensure optimal growth and survival of *Listeria monocytogenes* during different environmental stresses.

IV. Evidence of Resistance to Food Antimicrobials by Foodborne Pathogens

A. Food Preservative Antimicrobials

Food antimicrobials are chemical compounds used to extend the lag phase or kill microorganisms. They are added directly to food or, as sprays or dips for surface decontamination (Davidson and Harrison, 2003). Food antimicrobials may be classified into two groups: traditional or "regulatory approved" and naturally occurring (Davidson, 2001). Some traditional antimicrobials include acetic acid and acetates, sorbic acid and sorbates, benzoic acid and benzoates, lactic acid and lactates, propionic acid and propionates, and nitrites and nitrates (Table 1.0). Naturally occurring antimicrobials include compounds from microbial, plant and animal sources. A few, including lactoferrin, lysozyme, nisin and natamycin, which are approved in the United States and certain other countries for use in selected foods.

For many years, food antimicrobials were used primarily to prolong shelf life and preserve quality of foods through inhibition of spoilage microorganisms. Recently, food processors have been using antimicrobials more to inhibit or inactivate pathogenic microorganisms in foods. Historically, the only food antimicrobial used exclusively to inhibit a pathogen in a food is nitrite, which is used to inhibit *Clostridium botulinum* in cured meats (Davidson and Harrison, 2003). More recently antimicrobials such as lactates and diacetates have been added to processed meats or spray sanitizers on beef carcasses to inactivate pathogens such as *L. monocytogenes* (FDA, 2000).

Table 1.0 Some of the most common traditional and naturally occurring food antimicrobials approved by the Food and Drug Administration. (Adapted from Davidson and Harrison, 2002).

Antimicrobial compounds	Microbial target	Primary food application	Title 21 CFR designation
Acetic acid, acetates, diacetates	Yeasts, bacteria	Baked goods, meats & dairy products	184.1005, 182.6197, 182.1754, 184.1185, 184.1721, 172.130
Benzoic acid, benzoates	Yeasts, molds	Fruit beverages, baked goods & high acid foods	184.1021, 184.1733
Lactic acid, lactates	Bacteria	Meat, fermented foods	184.1061, 184.1207,184.1639, 184.1768
Lactoferrin	Bacteria	Meats	
Lysozyme	Clostridium botulinum and other bacteria	Cheese, hot dogs, cooked meats	184.1550
Nisin	Clostridium botulinum and other bacteria	Cheese	184.1538
Nitrite, Nitrate	Clostridium botulinum	Cured meats	172.160, 172.170, 172.175, 172.177
Propionic acid, propionates	Molds	Baked goods and dairy products	184.1081, 184.1221, 184.1784
Sorbic acid, sorbates	Yeast, molds, bacteria	Fruit beverages, baked and potato products, wines	182.3089, 184.3225, 182.3640; 182.3795

While food antimicrobials have been used for many years in the food industry, there are few data concerning the developed resistance by microorganisms to these antimicrobial compounds. Still, there are reasons to be concerned such as the increasing use of these compounds to inhibit or kill specific foodborne pathogens, and the increasing development and spread of therapeutic antibiotic-resistant microorganisms in the environment (Davidson and Harrison, 2002).

Unfortunately, there is little specific information about the mechanisms of action or resistance to most food antimicrobials. As with therapeutic antibiotics, resistance responses of microorganisms to food antimicrobials could be classified as either innate or acquired (Russell, 1991). Innate resistance is demonstrated by differences among related genera, species or strains of microorganisms under identical conditions of exposure (Davidson and Harrison, 2002). In food application of antimicrobials, apparent innate resistance also may be influenced by environment, food component interactions, processing interactions or presence of antagonistic inhibitors (Davidson and Harrison, 2002). Acquired resistance is not though to be a major problem with antimicrobials used in food against bacteria because, as opposed to antibiotics that have specific target site, food antimicrobials are more non-specific in their inhibition (Davidson and Harrison, 2002).

Fungi have been the most studied microorganisms associated with resistance to traditional food antimicrobials. This might be because many of the traditional food antimicrobials, e.g., benzoates and sorbates, are primarily used as antifungal agents. For example, Balatsours and Polymenacos (1963), isolated resistant yeasts from preserved

citrus beverages that contained 500 mg/liter of benzoic acid, and had a pH value of approximately 2.5. Other studies with noncarbonated orange drinks and reconstituted fruit juices showed that spoilage yeasts were not inhibited by the presence of benzoates or sorbates (McDonald 1963; King and Halbrook, 1987; Guerzoni et al., 1990).

Many yeasts species are relatively tolerant to weak-acid type preservatives. Some yeast that has been found to be resistant to weak organic acids includes *Zygosaccharomyces bailii*, *Saccharomyces cerevisiae* and *Candida krusei* (Warth, 1977). Certain strains of *Saccharomyces cerevisiae* will grow in the presence of up to 3 mM (0.03%) sorbic acid at pH 4.5, although the presence of the preservative causes both a drastic lag phase extension and a reduction of final biomass yield (Stratford and Anslow, 1996; Piper et al., 1998). Warth (1985) found that maximum tolerated levels of benzoic acid ranged from 0.05 mM (0.006%) for *Hansenula anomala* to 4 mM (0.05%) for *S. bailii* (*Zygosaccharomyces bailii*). The ability of certain yeasts species to grow at low pH in the presence of weak organic acid food preservatives enables them to act as important agents of food spoilage, which can cause considerable economic losses (Deak, 1991; Fleet, 1992).

Osmotolerant are yeasts are well known to be resistant to some food antimicrobials such as sorbates. Osmotolerant yeasts are able to grow at reduced water activities, such as those existing in presence of high sugar (>60%) concentrations. Although the general rule is that increasing sucrose concentrations (20-70%), decreasing pH, and increasing sorbate concentrations generally enhances inhibition of yeasts, osmotolerant yeasts are or more resistant to food antimicrobials such as sorbates under high concentrations of sucrose

(Sofos, 1989). Osmotolerant yeasts are mostly species of the genus *Saccharomyces* and *Zyosaccharomyces* and include *S. rouxii*, *S. bailii*, *S. bisporus* and *S. acidifaciens*.

The resistance of spoilage yeast to weak organic acids antimicrobials has been extensively studied and is known to depend on the H+ pumping P-type membrane ATPase system (Holyoak et al., 1996). Warth (1977) suggested that resistance to benzoic acid resulted primarily from an inducible energy-requiring system (H+ pumping proton-type membrane ATPase) that transported benzoate from the cells. Later, Warth (1989) stated that energy was required for reduction of cytoplasmic benzoate concentration and maintenance of internal pH of yeasts. Similarly, Warth (1989) found that *Z. bailii* in the presence of an energy source such as glucose, continuously eliminated benzoic acid from the cell. This maintained both a low intracellular concentration of the benzoate anion and a high intracellular pH, but at the expense of continuous expenditure of energy (Warth 1977; 1989). This system seems to be generated in many yeast species and is likely very important to cellular tolerance to a preservative (Warth, 1985).

Resistance among yeast species to benzoates has also been attributed to the rate of penetration into the cell by benzoic acid (Warth, 1989). For example, pumping benzoate anions out of the cell could create a futile cycle where anions re-associate at the lower external pH and reenter the cell (Brul and Coote, 1999). If altering membrane composition or structure could reduce the rate of diffusion across the plasma membrane, access to the cell interior by the toxic compound would be reduced (Brul and Coete 1999). Adapted cells could therefore have shorter lag times and show growth at higher benzoate levels than cultures not induced for the anion transport system (Warth, 1985).

Certain molds are also very resistant to common food antimicrobials. Schroeder and Bullerman (1985) found that two strains of *P. digitatum* and one strain of *P. italicum* developed increased tolerance to potassium sorbate and sorbic acid after they were repeatedly exposed to subinhibitory concentrations of the compounds. The MIC (minimum inhibitory concentration) of sorbic acid at pH 4.75 of *P. digitatum* was 1.78 mM (0.02%) to 2.23 mM (0.025%) and at pH 5.5 was 5.35 mM (0.06%) to 7.14 mM (0.08%). It appears that under certain conditions, some molds can grow and metabolize sorbate, especially *Penicillium* sp. Several studies have found that *Penicillium* strains isolated from cheeses treated with sorbate were able to grow and metabolize high concentrations (16 mM-17.8 mM) (0.18-0.20%) of sorbate (Marth et.al., 1996; Bullerman, 1977; Finol et al., 1982). In another study using Swiss cheese, 87% of isolates that tolerated potassium sorbate were *Penicillium* species and less than 1% were *Aspergillus* species (Liewen and Marth, 1985). Marth et al. (1996) demonstrated that some molds in the genus *Penicillium* could grow in the presence of up to 47.3 mM (0.71%) of potassium sorbate. These molds were isolated from natural and processed Cheddar cheese treated with sorbate.

There is little data on the resistance of foodborne bacteria to traditional food antimicrobials. Bacteria are quite variable in their resistance to food antimicrobials but some studies have found that the potential does exist for resistance development. Some bacteria that have been found to be resistant to organic acids including *E. coli*, *L. monocytogenes* and *S.* Typhimurium (Brul and Coote, 1999; Lin et al., 1996; Davis et al., 2002). Some foodborne pathogens when exposed to low pH via short chain organic acids

or inorganic acids (e.g., HCl) may undergo changes that provide them with varying degrees of resistance to subsequent exposure to normally lethal acidic conditions (Davidson and Harrison, 2002). This increased resistance to low pH through pre-exposure has been termed "acid habituation", "acid tolerance" or "acid shock". The practical importance of acid adaptation or tolerance by foodborne pathogens depends on the survival of the microorganism. Acid adapted or tolerant foodborne pathogens would have to possess enhanced survival in foods or food processing systems in which they are normally inactivated to be of importance (Davidson and Harrison, 2002).

Goodson and Rowbury (1989) found that cells of *E.coli* initially grown in media at pH 5.0 survived exposures to inorganic acid or to acid pH plus organic acid that prevented subsequent growth of cells at pH 7.0. Six organic acids, including benzoic, were evaluated; none of these acids inhibited subsequent growth of acid-adapted cells. They suggested that pre-exposure of the cells to the dissociated form of acid enabled some cells survive a lethal dose. Leyer et al., (1995) found that acid adapted (pH 5.0, 37°C for 4-5 hours) *E.coli* O157:H7 had greater survival than unadapted cells in acidified foods 125 mM or 1.12% lactic acid including salami or apple cider. Similarly, Garren et al., (1998) found that after acid adaptation (pH 5.5), strains of *E.coli* O157:H7 tolerated higher levels of sodium lactate (892 mM-2678 mM; 10-30%). Kwon and Ricke (1998) reported that *S.* Typhimurium had greatly enhanced acid resistance to high concentrations (100 mM) of acetate, butyrate and propionate (100 mM) when cells well pre-exposed to a mildly acid pH of 5.8.

Several research studies have demonstrated that acid adaptation or tolerance may produce pathogens with enhanced survival in foods to which a combination of antimicrobials have been added. For example, Kwon et al., (2000) found that *Salmonella* Typhimurium cells adapted by exposure to short chain fatty acid mixture (acetate 8 mM (0.1%), butyrate 3 mM (0.03%), lactate 14 mM (1.5%), propionate 2 mM (0.2%), succinate 9 mM (1.5%)) or propionic acid 0-200 mM (0-3.7%) alone had significantly increased resistance (>3-4 logs) to low pH (pH 3.0) for up to 3 h at 37°C in tryptic soy medium compare to unadapted cells. In another study also by Kwon and Ricke (1998; 2000), *S.* Typhimurium isolates tolerated various concentrations of propionic acid 0-200 mM (0-3.7%) under both anaerobic and aerobic conditions following acid adaptation (pH 3.0-5.0). In this study, resistance to the presence of propionic acid by *S.* Typhimurium was attributed to selective outgrowth of the mutant sub-populations or physiological adaptations, which allowed the cells to become more resistant to the acid (Kwon and Ricke, 1998).

Related to this, *Salmonella* serovars Typhimurium, Enteritidis, Heidelberg and Javiana that were pre-exposed to pH 5.8 showed increased resistance to the food antimicrobials such as lactic, propionic and acetic acid (Leyer and Johnson, 1992). Gahan et al., (1996) demonstrated that by adapting *L. monocytogenes* LO28 to lactic acid at pH 5.5 for 60 min, cells had enhanced survival in low acid foods such as yogurt, cottage cheese, orange juice and salad dressings. Many of these studies have found that increased resistance was dependent upon the strain and environmental conditions (i.e. pH, growth medium). In contrast, Pickett and Murano (2001) found no differences in susceptibility of

Listeria monocytogenes cells pre-exposed to sublethal levels of lactic acid 22.2 mM (0.2%), citric acid 10.4 mM (0.2%) and propionic acid 5.36 mM (0.1%) at pH 2.8 for up to 60 minutes at 37°C before challenging the cells to minimum inhibitory concentrations. Lu et al., (2005) reported that *L. monocytogenes* survived in refrigerated storage in the presence of 6.0% sodium diacetate

Different species and strains of bacteria exhibit varying sensitivity to inhibition by sorbate (Sofos, 1989). *Staphylococcus* spp. have been reported to be the most resistant bacteria to sorbate followed by *Pseudomonas* spp., *Acinetobacter* spp., *Alteromonas* (*Pseudomonas*) *putrefaciens*, yeasts, and *Moraxella* spp. (Sofos, 1989). In one study, *Staphylococcus aureus* was resistant to 446 mM (5.0%) sorbate at pH 5.0–7.0 (Lahellec et al., 1981). However, other studies have shown that 8.9-44.6mM (0.1-0.5%) sorbate inhibited growth, thermonuclease and enterotoxin production by *S. aureus* in several products, including minced cod, bacon, uncured sausage, and cooked turkey meat (Lynch and Potter, 1982; Pierson et al., 1979; Tompkin et al., 1974; To and Robach, 1980). Normally, *Clostridium botulinum* is sensitive to sorbate (Sofos et al., 1979). However, germination of spores of certain strains of *Clostridium botulinum* may not be inhibited by sorbate (Sofos et al., 1979). Significant differences existed in a study among ten strains of *C. botulinum* spores exposed to 23.2 mM (0.26%) sorbate at pH 5.65 at 37°C and most strains were tolerant to sorbate (Blocher et al., 1982). Banerjee and Sarkar (2004) found that *Clostridium perfringens* was not inhibited at 17.8 mM (2.0%) sorbic acid (pH 6.50) and at 15.6-18.0 mM (1.9-2.2%) benzoic acid (pH 6.47-6.27).

Use of organic acid sprays as sanitizing treatments for meat carcasses has become very common (Dickson, 1995). Spraying with organic acid solutions and/or hot or cold water (Hardin et al., 1995; Sofos and Smith, 1998) has been increasingly applied as a sequential intervention for meat decontamination (Bacon et al., 2000). These interventions can significantly reduce (i.e. 1-3 logs) microbial contamination on meat carcass surfaces (Hardin et al., 1995; Sofos and Smith, 1998). It has been suggested that the mixing of organic acids in spray runoff from the meat in packing plants has the potential to exert a sublethal acid stress on meatborne pathogens (Samelis et al., 2001a, 2002). This may result in biofilms composed of acid stressed and /or adapted pathogenic strains. Extended acid stressing also may enhance virulence and trigger adaptive mutations of permanent acid stress resistance (Archer, 1996; Sheridan and McDowell, 1998). Related to this, Stopforth et al. (2003) found that acid-adapted *E.coli* O157:H7 and acid-adapted *L. monocytogenes* survived when inoculated into water/organic acid washings stored for up to 14 days at 15°C with densities ranging from 6.7-7.3 and 6.4-6.7 log cfu ml, respectively. The strains were previously acid-adapted by growing each at pH 4.4-4.5 for *L. monocytogenes* and at pH 5.0-5.1 for *E. coli*. Several washings were used including composite of water washings, three dilutions (1/9, 1/49, 1/99 v/v) of the 222 mM lactic acid (2%) and same three dilutions for 140 mM acetic acid (2%) (Stopforth et al., 2003). The pH of each organic acid washing was measured during storage to understand the behavior of pathogens at different pH levels. Overall, the lower the concentration of acid (1/9 v/v) and the higher the pH (6.0) in the washing mixtures (both acetic and lactic acid), the better the survival of both pathogens during storage at 15°C,

with *E. coli* O157:H7 being more acid-tolerant than *L. monocytogenes* from day 0, and more resistant to acetic acid than to lactic acid.

V. Methodology to Evaluate Resistance

There are many methods used to determine antimicrobial effectiveness of therapeutic antibiotics, sanitizers and food antimicrobials. With antibiotics, bacterial susceptibility is determined mainly by the agar diffusion or agar broth dilution assays (Russell, 2002). Sanitizers or biocides are mainly evaluated with standardized assays (Russell, 2002).

Methods for evaluating food antimicrobials have been in existence nearly as long as those for sanitizers and therapeutic antibiotics (Lopez-Malo et al., 2005). The most common current methods used to evaluate the efficacy of food antimicrobials are *in vitro* and application methods. The *in vitro* methods can give preliminary information to determine potential usefulness of the test compound in a food (Lopez-Malo et al., 2005). In this case the antimicrobial compound in not applied directly into the food product. *In vitro* methods are classified as endpoint and descriptive. In endpoint methods microorganisms are challenged for an arbitrary period of incubation time and qualitative information of the inhibitory power of the antimicrobial compound is obtained at the end of the period testing (Lopez-Malo et al., 2005). These include methods such as agar diffusion, agar and broth dilution, gradient plates, spiral plating and sanitizer and disinfectant tests (Davidson and Parish, 1989).

In descriptive methods, microbial growth is measured over time to obtain quantitative information about the growth dynamics (Lopez-Malo et al., 2005). Descriptive methods include methods such as turbidimetric assays and inhibition curves.

Application methods are those in which an antimicrobial is applied directly to a food product to determine its effectiveness on natural microflora (Lopez-Malo et al., 2005).

Applied tests are used in actual foods to evaluate some factors that can affect the efficacy of natural antimicrobials and these include endpoints tests and inhibition curves methods (Davidson and Parish, 1989; Lopez-Malo et al., 2000a).

Any method used to evaluate antimicrobial efficacy will be greatly affected by factors such as the test microorganism, antimicrobial agent, test medium, and test procedure itself (Lopez-Malo et al., 2005). One of the most important factors is the initial number and physiologic state of the test microorganism. This is because inocula too concentrated or too diluted can result in false-resistant and false-sensitive results, respectively (Lopez-Malo et al., 2005). Growth phase is also very important since log-phase cells are more sensitive than stationary-phase vegetative cells.

A. Agar Diffusion Assays

Agar diffusion assays were one of the first assay methods developed in the 1940's. They involve diffusion of compounds in agar from wells or paper disks (Piddock, 1990). In 1966, Bauer et al. proposed a standardized disk susceptibility method. Today, in the United States, the National Committee for Clinical Laboratory Standards (NCCLS) publishes standardized procedures for agar diffusion and dilution assays for the activity of antibiotics and the bactericidal activity of antimicrobial agents (NCCLS. 1999, 2002). In this test, the antimicrobial compound diffuses through the agar resulting in a concentration that is inversely proportional to the distance from the disk or well (Figure 3.0). A zone of no growth around the disk or well indicates the degree of inhibition and it is dependent on the rate of diffusion of the compound and the cell growth (Barry, 1986).

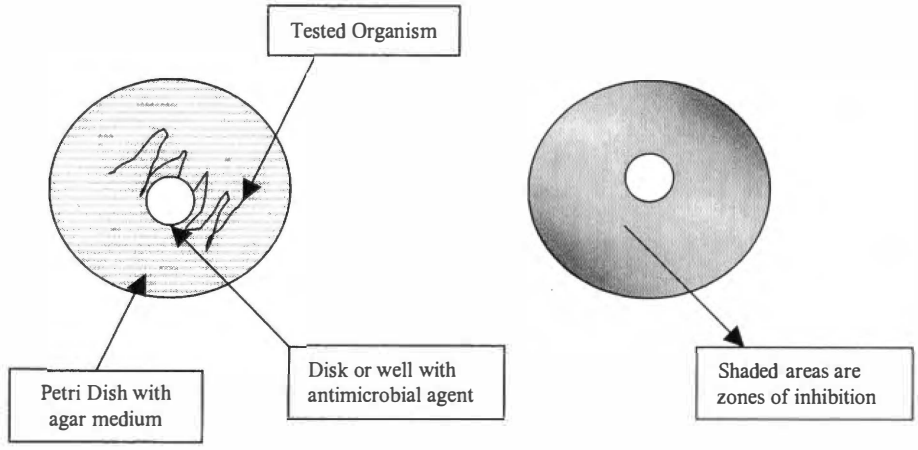

Figure 3.0 Determination of the "zone of inhibition" by paper disk or well diffusion method. (Adapted from Davidson et al., 2005)

In the agar diffusion test, Petri dishes are prepared with a non-selective medium and the surface is inoculated with approximately 6 logs CFU/ml over the entire agar surface. After inoculation the surface is allowed to dry for 10 min. After incubation, growth of the organism and diffusion of the antimicrobial agent results in a circular zone of inhibition in which the amount of antimicrobial exceeds inhibitory concentrations (Barry, 1986). Results in the agar diffusion are generally qualitative and zone diameters are termed as susceptible when the zone is >30 to 35 mm in diameter, intermediate with a zone of 20 to 30 mm, or resistant with a zone of <15 to 20 mm (Piddock, 1990; Ginocchio 2002). This test must be carefully standardized since the zone size is also dependent on the inoculum size, medium composition, temperature of incubation, excess moisture, and thickness of agar (Langsrud and Sundheim, 1998).

B. Double Gradient/Wedge Assay

Gradient or wedge plating is a method to observe relative sensitivity among several microorganisms on agar medium (Lopez-Malo et al., 2005). For this method, Petri dishes plates are prepared with two gradient layers, the top one containing approximately 15ml of non-selective agar medium and the bottom layer containing another 15 ml of the medium and the antimicrobial agent. This method of antimicrobial testing can be semi-quantitative and factors such as incubation time, inoculum preparation, and initial number must be consistent for reproducible results (Lopez-Malo et al., 2005). Bala and Marshall (1996) developed double gradient technique to evaluate influence of NaCl and monolaurin on inhibition of *L. monocytogenes*. Thomas et al., (1992) also used gradient plating techniques to evaluate influence of NaCl, pH and temperature-pH on the growth of mixed inoculum of six *Salmonella* strains. Gradient plating techniques have also been used to evaluate the synergistic effect of sucrose fatty acid esters and nisin on inhibition of *L. monocytogenes*, *Bacillus cereus*, *Lactobacillus plantarum* and *S. aureus* (Thomas et al., 1998).

C. Agar/Broth Dilution Assays

Agar or broth dilution assays are used to quantitatively determine minimum inhibitory concentrations, whether an antimicrobial is lethal to a test microorganism, for organisms with a variable growth rate, and for anaerobic or microaerophilic microorganisms (Barry, 1996). For both methods, a single statistic known as minimum inhibitory concentration (MIC) is generated to describe the inhibition. The MIC defined

as the lowest concentration that results in no growth after a specified incubation period (Carson et al., 1995; Lambert and Pearson, 2000).

An agar dilution assay is performed by adding 15 ml of a nonselective agar into a Petri plate with a diluted concentration of the antimicrobial compound. The concentration of antimicrobial is normally determined to provide a realistic MIC (Lopez-Malo et al., 2005). Then plates are allowed to dry to be then inoculated with the test microorganisms. The test microorganism is diluted to around 7.0 log CFU/ml added to plates in 1-2 μl spots (around 4 log CFU/ml per spot), and plates are incubated at the optimum temperature of the test microorganism (NCCLS, 1999, 2002). The MIC is defined as the lowest concentration that prevents growth of the inoculum on the agar surface.

In the broth dilution assay, an antimicrobial is serially diluted and a single concentration is added to a culture tube of nonselective broth medium and inoculated with approximately 5-6 log CFU/ml of the test microorganism (Lopez-Malo et al., 2005; Thrupp, 1986; Piddock, 1990; NCCLS, 1999). The micro-broth dilution assay is the same as the broth dilution assay but is carried out in microtiter plate. Wells are filled with approximately 50-100 μl broth plus a single concentration of antimicrobial and 4.7 CFU/ml of inoculum per well. The remainder of the assay is the same as the standard broth dilution assay (Lopez-Malo et al., 2005). For the broth and microbroth dilution assays, the minimum bactericidal concentration (MBC) or minimum lethal concentration (MLC) is the lowest concentrations of antimicrobial that produces 99.9% kill of the test microorganism (NCCLS, 2002). This is performed following incubation of the tubes or plates. A portion of the wells or tubes in which the microorganism exhibits no growth (no

turbidity) is plated on a nonselective agar using spread plate method (Lopez-Malo et al., 2005).

VI. Objectives of the Current Studies

The aim of this study is to investigate the potential development of resistance to regulatory approved food antimicrobials by *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* after repeated exposures to food antimicrobials and grown in different medium environments. The food antimicrobials used in this study include potassium sorbate, sodium benzoate, sodium lactate and sodium diacetate. The following microorganisms were all used: *Listeria monocytogenes* strains 101, 108, 310, and Scott A and *Salmonella enterica* serovar Typhimurium strains ATCC 2380, ATCC 2576, ATCC 2582 and ATCC 2486.

Cells were not subjected to any pre-stress condition. An agar dilution assay was performed to determine susceptibility and adaptation to increasing antimicrobial concentrations. A microbroth dilution assay was used to determine tolerance development after two consecutives exposures to same antimicrobial concentrations, and one further exposure to a step higher concentration. Once cells were adapted to the highest concentration, cells were grown in two different media to determine if the presence of glucose affected resistance to antimicrobials.

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PART TWO: POTENTIAL DEVELOPMENT OF RESISTANCE TO FOOD ANTIMICROBIALS BY *LISTERIA MONOCYTOGENES* AND *SALMONELLA* TYPHIMURIUM

Abstract

The potential for development of resistance of four strains of *Listeria monocytogenes* (101, 108, 310 and Scott A) and *Salmonella* Typhimurium DT 104 (2380, 2576, 2582, 2486) to the food antimicrobials sodium benzoate (SB), potassium sorbate (PS), sodium diacetate (SD) and sodium lactate (SL) at pH 6.0 was studied. Cells were not subjected to any pre-stress condition. An agar dilution assay was performed to determine susceptibility and adaptation to increasing antimicrobial concentrations. A microbroth dilution assay was used to determine tolerance development after two consecutives exposures to same antimicrobial concentrations, and one further exposure to a step higher concentration. In the agar dilution assay, the initial minimum inhibitory concentrations (MIC) for *L. monocytogenes* were 0.3%, 0.2-0.3%, 2.0% and 0.5% (all w/v), for PS, BS, SL and SD, respectively. For *S.* Typhimurium, initial MICs were 0.3%, 0.3-0.4%, 2.0% and 0.5%, for PS, SB, SL and SD, respectively. Both microorganisms demonstrated increased resistance after growth in the presence of the food antimicrobials up to 0.5% PS, 4.0% SL and 1.0% SD. For SB, *Listeria* cells adapted up to 0.4% (except strain 108) and *Salmonella* cells to up to 0.5%. Using a microbroth dilution assay, all strains showed less susceptibility to low levels (0.1-0.3%) of benzoate and potassium sorbate. There was no significant increase in OD of culture suspensions that contained 0.4-0.5% of benzoate or potassium sorbate after initial, second and third exposures (except for *L. monocytogenes* 310). Results indicated that 4.0% SL had essentially no effect on any cell growth. For all strains there was no change in absorbance (OD_{630}) at

>1.0% SD. Both microorganisms showed potential for resistance to SL and to lower concentrations of BS and PS at certain pH.

I. Introduction

In the food industry, antimicrobial food preservatives have been used for decades to control bacterial growth in order to prolong shelf-life and preserve food quality by inhibiting growth of food spoilage microorganisms. In recent years, antimicrobial food preservatives have been increasingly utilized to improve food safety by inhibiting foodborne pathogens such as *Listeria monocytogenes*, *Salmonella,* and *E. coli* O157:H7. While antimicrobial food preservatives have been safely used for many years in the food industry to extend shelf life of foods, there are few data concerning the potential for developed resistance by target microorganisms to these compounds. If antimicrobial food preservatives are to be utilized as a major part of a pathogen control system, information on the potential for resistance development must be investigated (Davidson and Harrison, 2002).

Evaluating the potential for resistance development to antimicrobials food preservatives stems from the increased incidence of microorganisms exhibiting resistance to antibiotics used for therapeutic purposes in human and animal medicine. According to the Centers for Disease Control and Prevention (CDC), approximately 70% of bacteria causing hospital infections are now resistant to at least one antibiotic. The increased number of multidrug resistant microorganisms in clinical and farm settings has been mainly attributed to the proliferation of resistance genes and to the ease of dissemination of resistant strains between humans and animals especially via food of animal origin (Aarestrup, 1999; van den Bogaard and Stobberringh, 1999). Broad use of antibiotics has

created both a strong selective pressure, which results in the survival, and spread of resistant bacteria, as well as increased the potential for the development of resistance to other antimicrobial agents.

There is little specific information about the mechanisms of action or resistance to most food antimicrobials. As with therapeutic antibiotics, resistance responses of microorganisms to food antimicrobials could be classified as either innate or acquired (Russell, 1991). Innate resistance is a chromosomally controlled property associated with the microorganism. Since food antimicrobials have a generally broad spectrum, it is believed that resistance is most likely due to unspecified reduced uptake controlled primarily by innate characteristics within the organisms (Russell 1991; Russell et al., 1997; Fernandes, 2003). The primary mechanisms associated with intrinsic or innate resistance include microbial cell impermeability, inactivation of toxic compounds via microbial enzymes and efflux pumps (Davidson and Harrison, 2003). Innate resistance is demonstrated by differences among related genera, species or strains of microorganisms under identical conditions of exposure (Davidson and Harrison, 2002). In food application of antimicrobials, apparent innate resistance also may be influenced by environment, food component interactions, processing interactions or presence of antagonistic inhibitors (Davidson and Harrison, 2002).

Acquired resistance results from genetic changes in the microbial cell through direct mutation of the chromosome acquisition of plasmids, transposons, or other genetic elements (McDonnell and Russell, 1999; Russell 1991; 2003). Acquired resistance occurs primarily among bacteria and toward antibiotics. This is because antibiotics used for

therapeutic purposes generally have specific target sites in microbial cells and therefore have greater selective pressure for development of acquired resistance (Davidson and Harrison, 2002). Acquired resistance is not though to be a major problem with antimicrobials used in food against bacteria because, as opposed to antibiotics, which have specific target site, food antimicrobials are more non-specific in their inhibition (Davidson and Harrison, 2002).

Some foodborne pathogens when exposed to low pH via short chain organic acids or inorganic acids (e.g., HCl) may undergo changes that provide them with varying degrees of resistance to subsequent exposure to normally lethal acidic conditions (Davidson and Harrison, 2002). This increased resistance to low pH through pre-exposure has been termed "acid habituation", "acid tolerance" or "acid shock". The practical importance of acid adaptation or tolerance by foodborne pathogens depends on the survival of the microorganism. Acid adapted or tolerant foodborne pathogens would have to possess enhanced survival in foods or food processing systems in which they are normally inactivated to be of importance (Davidson and Harrison, 2002). While resistance development has been demonstrated for cells pre-exposed to stress, there is little or no data on adaptation by foodborne pathogens to antimicrobial food preservatives in the absence of such stresses.

Therefore, the purpose of this study was to investigate the potential for foodborne pathogens to become resistant to traditional, regulatory-approved food antimicrobials without any pre-stress.

II. Materials and Methods

A. Bacterial Isolates

Listeria monocytogenes strains 101, 108, 310, and Scott A and *Salmonella* Typhimurium DT104 strains 2380, 2576, 2582 and 2486 (animal isolates originally isolated from animal sources by D. Hancock and T. Besser, College of Veterinary Medicine, Washington State University, Pullman, WA) were obtained from the University of Tennessee, Department of Food Science and Technology culture collection. *L. monocytogenes* and *S.* Typhimurium were grown in trypic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) with 0.6% yeast extract (Difco), TSBYE, at 32°C. Agar media for *L. monocytogenes* and *S.* Typhimurium were tryptic soy agar (TSA; Difco). Cultures were maintained on TSA slants at 4°C and transferred monthly to maintain viability. A working culture was prepared by inoculating a loopful of culture into 9 ml of TSB. The culture was then subjected to two successive 24 h transfers before being used.

B. Food Preservative Antimicrobials

Potassium sorbate (Acros Organics, Geel, Belgium), sodium benzoate (Sigma-Aldrich Chemicals, St. Louis, MO), sodium diacetate (Sigma-Aldrich Chemicals) and sodium lactate (Purac America, Lincolnshire, IL) were used. Stock solutions were prepared with 25 g of each antimicrobial in 100 ml of deionized water. Stock solutions were mixed thoroughly and then filter-sterilized using 0.45-μm-membrane filter (Millipore, Bedford, MA.). Fresh stock solutions were prepared weekly and stored at 4°C until use.

C. Agar Dilution Assay

An agar dilution assay was performed to determine susceptibility and adaptation of the test strains to antimicrobials used. First, susceptibility of *L. monocytogenes* and *S.* Typhimurium isolates to food preservatives antimicrobials was determined by spreading 0.5 µl from a 24 h culture (8-9 log CFU/ml) onto15 ml TSA. Petri plates were prepared containing a range of different antimicrobial concentrations. Plates were incubated at 32°C for 48 h. Secondly, potential for adaptation of *L. monocytogenes* and *S.* Typhimurium isolates was determined by exposing strains to increasing antimicrobial concentrations every 48 h. An initial 0.5 µl from a 24 h culture (8-9 log CFU/ml) was spread plated onto 15 ml TSA containing the initial lowest antimicrobial concentration and plates incubated at 32°C. If growth was observed, a loopful (10 µl) of culture was transferred to another plate containing a higher antimicrobial concentration. Strains were exposed to higher concentrations until growth was inhibited or strains became more susceptible to the antimicrobial. Potassium sorbate and sodium benzoate were in the range of 0.1-0.6% (w/v), sodium lactate at 0.5-4.0% (w/v), and sodium diacetate 0.5%-2.0% (w/v). The pH of the media was adjusted to 6.0 with sterile 1N HCl before pouring plates. Growth observed was noted as heavy, moderate, visible, hazy or no growth. The minimum inhibitory concentrations (MICs) were defined as the lowest concentration at which growth was completed inhibited (hazy or no growth) after 48 h. Where microbial growth occurred at the highest concentration of antimicrobial exposure, cells were re-grown on TSA slants in the presence of that concentration and incubated at 32°C for 48 h.

These strains were considered "adapted" or "resistant" to the highest antimicrobial concentration used.

To determine if "adapted" strains lost viability and/or adaptation upon prolonged storage under same highest concentration, TSA slants were stored at 4°C for up to 3 months. Cells were re-grown by transferring a loopful of growth from one slant to a TSA plate containing same concentration at which the cells were previously exposed. Plates were incubated at 32°C for 48h and growth observed as noted previously.

D. Microbroth Dilution Assay

A microbroth dilution assay was used to determine the potential for development of tolerance or adaptation to the antimicrobials over time. Sterile 96-well microtiter plates (Fisher Scientific, Hampton, NH) with a well capacity of 300 µl were used. Microtiter plates wells were filled with 125 µl of double-strength TSB (Difco), 100 µl of antimicrobial solutions, and 50 µl of inoculum (approximately 10^7-10^9 CFU/ml) for a total volume of 250 µl. Potassium sorbate, sodium benzoate and sodium lactate were in the range of 0.1-0.6% (w/v) and sodium diacetate 0.5%-3.0% (w/v). The pH of each media was adjusted to 6.0 with sterile 1N HCl. Microtiter plates were incubated at 32°C for 24 h. The optical density at 630nm (OD_{630}) was monitored at 0, 6, 12 and 24 h using an Elx800 Universal Microplate reader (Biotek Instruments Inc., Winooski, VT). After exposure to the concentration of antimicrobial, cells were re-exposed to the same initial concentration by transferring 50 µl of culture (approximately 10^5-10^7 CFU/ml) to a second microtiter well filled with 125 µl of double-strength TSB (Difco) and 100 µl of

antimicrobial solutions. Following incubation with the second exposure, cells were transferred to a third higher concentration to determine tolerance. As with the second exposure, cells were re-exposed by transferring (50 μl; approximately 10^5-10^7 CFU/ml) to a microtiter plate well filled with 125 μl of double-strength TSB(Difco) and 100 μl of a higher concentration of antimicrobial solution. The purpose of a second and third exposure was to determine if *Listeria* and *Salmonella* strains developed any levels of tolerance upon repeated exposures of antimicrobials at different concentrations. All samples were run in duplicates.

III. Results

Using an agar dilution type, susceptibility of parent cells was determined by exposure to a range of antimicrobial concentrations. This was used as an indication of tolerance of each strain tested when exposed initially to different antimicrobial concentrations at pH 6.0 without any pre-stress. Results indicated that *L.* monocytogenes grew well up to 0.2%-0.3% potassium sorbate and *S*almonella Typhimurium grew up to 0.3% potassium sorbate and two strains (2576 and 2582) grew at the maximum of 0.4% sodium benzoate (Table 1.0 and 2.0). All *Listeria* and *Salmonella* strains tolerated levels up to 2.0% sodium lactate (Table 3.0) and 0.5% sodium diacetate (Table 4.0).

To determine if adaptation could occur, cells were exposed gradually to increasing concentrations every 48 h until growth was inhibited or strains became more susceptible to the antimicrobial. Most cells tolerated at least one higher concentration of the antimicrobial than their initial MIC to which they were previously susceptible. *Listeria* strains tolerated up to 0.5%, potassium sorbate (Table 5.0) and 0.4% sodium benzoate (Table 6.0) except strain 108 that had no increased tolerance to sodium benzoate. All *Salmonella* strains tolerated up to 0.5% of both antimicrobials (Table 5.0 and 6.0). All *Salmonella* Typhimurium except 2380 also grew at 0.6% potassium sorbate (Table 5.0) When exposed to increasing concentrations, all strains of both *L. monocytogenes* and *S.* Typhimurium tolerated up to 4.0% lactate (Table 7.0) and 1.0% diacetate (Table 8.0).

Table 1.0 Growth of *Listeria monocytogenes* and *Salmonella* Typhimurium strains in different concentrations of potassium sorbate (PS) at pH 6.0 in 48 h.

Strains	**Potassium Sorbate %**					
	0.1	**0.2**	**0.3**	**0.4**	**0.5**	**MIC[a]**
L. monocytogenes						
101	++	++	++	+/-	+/-	0.3%
108	++	++	++	+/-	+/-	0.3%
310	++	++	++	+/-	+/-	0.3%
Scott A	++	++	++	+/-	+/-	0.3 %
S. Typhimurium						
2380	+++	+++	+++	+/-	+/-	0.3%
2486	+++	+++	+++	+/-	+/-	0.3%
2576	+++	+++	+++	+/-	+/-	0.3%
2582	+++	+++	+++	+/-	+/-	0.3%

+++ Heavy, ++Moderate, +Visible, +/-Hazy, -Negative
[a] Minimum inhibitory concentration

Table 2.0 Growth of *L. monocytogenes* and *S.* Typhimurium strains to different concentrations of sodium benzoate (SB) at pH 6.0 in 48 h.

Strains	**Sodium Benzoate %**					
	0.1	**0.2**	**0.3**	**0.4**	**0.5**	**MIC[a]**
L. monocytogenes						
101	+++	+++	+++	+/-	+/-	0.3%
108	+++	+++	+++	+/-	+/-	0.3%
310	+++	+++	+/-	+/-	+/-	0.2%
Scott A	+++	+++	+/-	+/-	+/-	0.2%
S. Typhimurium						
2380	+++	+++	+++	+/-	+/-	0.3%
2486	+++	+++	+++	++	+/-	0.4%
2576	+++	+++	+++	++	+/-	0.4%
2582	+++	+++	+++	+/-	+/-	0.3%

+++ Heavy, ++Moderate, +Visible, +/-Hazy, -Negative
[a] Minimum inhibitory concentration

Table 3.0 Growth of *L. monocytogenes* and *S.* Typhimurium strains to different concentrations of sodium lactate (SL) at pH 6.0 in 48 h exposure.

Strains	**Sodium Lactate %**				
	0.5	**1.0**	**2.0**	**3.0**	**MIC[a]**
L. monocytogenes					
101	+++	+++	+++	-	2.0%
108	+++	+++	+++	-	2.0%
310	+++	+++	+++	-	2.0%
Scott A	+++	+++	+++	-	2.0%
S. Typhimurium					
2380	+++	+++	+++	-	2.0%
2486	+++	+++	+++	-	2.0%
2576	+++	+++	+++	-	2.0%
2582	+++	+++	+++	-	2.0%

+++ Heavy, ++Moderate, +Visible, +/-Hazy, -Negative
[a] Minimum inhibitory concentration

Table 4.0 Growth of *L. monocytogenes* and *S.* Typhimurium strains to different concentrations of sodium diacetate (SD) at pH 6.0 in 48 h exposure.

Strains	**Sodium Diacetate %**			
	0.5	**1.0**	**2.0**	**MIC[a]**
L. monocytogenes				
101	+++	-	-	0.5%
108	+++	-	-	0.5%
310	++	-	-	0.5%
Scott A	++	-	-	0.5%
S. Typhimurium				
2380	+++	-	-	0.5%
2486	+++	-	-	0.5%
2576	+++	-	-	0.5%
2582	+++	-	-	0.5%

+++ Heavy, ++Moderate, +Visible, +/-Hazy, -Negative
[a] Minimum inhibitory concentration

Table 5.0 Growth of *Listeria monocytogenes* and *Salmonella* Typhimurium strains to increasing concentrations of potassium sorbate (PS) at pH 6.0 in 48 h.

Strains	**Potassium Sorbate %**						
	0.1	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**MIC[a]**
L. monocytogenes						n/a	
101	+++	+++	+	+	+		0.5%
						n/a	
108	+++	+++	++	+/-	+		0.5%
						n/a	
310	+++	+++	++	++	+		0.5%
						n/a	
Scott A	+++	+++	++	++	+		0.5 %
S. Typhimurium							
2380	+++	+++	+++	+++	+	+/-	0.5%
2486	+++	+++	+++	+++	++	+	0.6%
2576	+++	+++	+++	+++	++	+	0.6%
2582	+++	+++	+++	+++	++	+	0.6%

+++ Heavy, ++Moderate, +Visible, +/-Hazy, -Negative n/a no exposure
[a] Minimum inhibitory concentration

Table 6.0 Growth of *L. monocytogenes* and *S.* Typhimurium strains to increasing concentrations of sodium benzoate (SB) at pH 6.0 in 48 h.

Strains	**Sodium Benzoate %**						
	0.1	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**MIC[a]**
L. monocytogenes							
101	+++	+++	+	+	-	n/a	0.4%
108	+++	+++	++	+/-	-	n/a	0.3%
310	+++	+++	++	++	-	n/a	0.4%
Scott A	+++	+++	++	++	+/-	n/a	0.4 %
S. Typhimurium							
2380	+++	+++	+++	++	+	+/-	0.5%
2486	+++	+++	+++	+++	+	-	0.5%
2576	+++	+++	+++	+++	++	-	0.5%
2582	+++	+++	+++	+++	+	+/-	0.5%

+++ Heavy, ++Moderate, +Visible, +/-Hazy, -Negative n/a no exposure
[a] Minimum inhibitory concentration

Table 7.0 Growth of *Listeria monocytogenes* and *S.* Typhimurium strains to increasing concentrations of sodium lactate (SL) at pH 6.0 in 48 h.

Strains	**Sodium Lactate %**				
	1.0	**2.0**	**3.0**	**4.0**	**MIC[a]**
L. monocytogenes					
101	+++	+++	+++	+++	4.0%
108	+++	+++	+++	+++	4.0%
310	+++	+++	+++	+++	4.0%
Scott A	+++	+++	+++	+++	4.0%
S. Typhimurium					
2380	+++	+++	+++	+++	4.0%
2486	+++	+++	+++	+++	4.0%
2576	+++	+++	+++	+++	4.0%
2582	+++	+++	+++	+++	4.0%

+++ Heavy, ++Moderate, +Visible, +/-Hazy, -Negative n/a no exposure
[a] Minimum inhibitory concentration

Table 8.0 Growth of *L. monocytogenes* and *S.* Typhimurium strains to increasing concentrations of sodium diacetate (SD) at pH 6.0 in 48 h.

Strains	**Sodium Diacetate %**			
	0.5	**1.0**	**2.0**	**MIC[a]**
L. monocytogenes	+++	+	-	
101				1.0%
	+++	+	-	
				1.0%
108				
	++	+	-	
310				1.0%
	++	++	-	
Scott A				1.0%
S. Typhimurium				
	+++	+	-	
2380				1.0%
	+++	+++	-	
2486				1.0%
	+++	+++	-	
2576				1.0%
	+++	+/-	-	
2582				1.0%

+++ Heavy, ++Moderate, +Visible, +/-Hazy, -Negative
[a] Minimum inhibitory concentration

Cells growing at the highest concentration of various antimicrobials were considered "adapted" or "resistant" strains. Cell viability was evaluated upon prolonged storage (3 months at 4°C) with highest concentration of antimicrobial. Strains did not lose viability and showed the same or slightly greater tolerance to the antimicrobials than the original MIC (Table 9.0). Overall, *L. monocytogenes* Scott A and 310 and *S.* Typhimurium 2582 and 2576 were the most resistant strains in the agar dilution assay.

In the microbroth dilution assay, adaptation or tolerance of parent cells to the antimicrobials was determined by exposing the cells twice to a single concentration of an antimicrobial and monitoring growth with optical density over time followed by exposure a third time to a higher concentration of the same antimicrobial. In the presence of potassium sorbate at pH 6.0, *Listeria monocytogenes* strains demonstrated growth up to 0.5% but final growth level decreased with increasing concentrations (Appendix 2.0, 6.0, 10.0, 14.0). Even at the lowest concentration (0.1%), growth was less than the control (Appendix 1.0). Re-exposure of the strains to the same concentration caused only a slight reduction in the final growth level at 24 hr with the exception of strain Scott A at > 0.3%. Exposure of the cells a third time at a concentration 0.1% higher than the first two exposures had variable results. Strains 310 demonstrated equivalent growth at all concentrations while strains 101, 108 and Scott A all showed little or not growth at 0.6% potassium sorbate.

Table 9.0 Growth of *L. monocytogenes* and *S.* Typhimurium strains upon prolonged storage (3 months 4°C) with the highest antimicrobial concentrations for sodium benzoate (SB), potassium sorbate (PS), sodium lactate (SL) and sodium diacetate (SD) at pH 6.0 in 48h.

Strains	**Antimicrobials %**			
	PS 0.4	**BS 0.4**	**SL 3.0**	**SD 1.0**
L. monocytogenes 101	+	+++	+++	+
108	+	+++	+++	+
310	+	++	+++	+
Scott A	+	++	+++	+
S. Typhimurium 2380	++	++	+++	+
2486	++	++	+++	+
2576	++	++	+++	+
2582	++	++	+++	+

+++ Heavy, ++Moderate, +Visible, +/-Hazy, -Negative
[a] Minimum inhibitory concentration

Based on optical density, sodium benzoate was more inhibitory to *L. monocytogenes* strains than potassium sorbate (Table 10; Appendix 3.0, 7.0, 11.0, 15.0). Little growth was demonstrated with any of the strains at ≥0.3%. Transfer, even at the same concentration generally had no effect or decreased growth further. A third transfer to a higher concentration appeared to allow growth to a slightly higher level at concentrations of sodium benzoate to 0.2%. With sodium lactate, results were variable (Table 10, Appendix 4.0, 8.0, 12.0, 16.0). Strain 108 showed little inhibition up to 4% and had slightly better growth upon exposure to the same concentration. Strains 108, 310 and Scott A showed moderate growth up to 4% sodium lactate and re-exposure caused slightly less or no difference in growth. Sodium diacetate was highly inhibitory to all strains except 101 at ≥1% (Table 10, Appendix 5.0, 9.0, 13.0, 17.0). Re-exposure to the compound generally reduced growth of all strains at all concentrations.

Salmonella Typhimurium was inhibited by potassium sorbate to a greater extent than was *Listeria monocytogenes* (Table 11, Appendix 18.0, 22.0, 26.0, 30.0). Only strain 2380 increased appreciably in OD at up to 0.4% potassium sorbate. None of the strains grew upon a second or third exposure to potassium sorbate. Similarly, sodium benzoate was highly inhibitory to *Salmonella* Typhimurium (Table 11, Appendix 19.0, 23.0, 27.0, 31.0). Little or no increase in OD was demonstrated even at 0.1-0.2% sodium benzoate with any of the strains. In contrast, sodium lactate had little effect on growth as measured by increased optical density even at 4% (Table 11, Appendix 20.0, 24.0, 28.0, 32.0). At 1% sodium lactate, transfer into the same concentration caused an increase in optical density while higher concentrations demonstrated slightly less growth. Sodium diacetate

Table 10.0 Change in optical density (OD) for *Listeria monocytogenes* strains at 24 hr after repeated exposure to different antimicrobial concentrations of potassium sorbate, sodium benzoate, sodium lactate and sodium diacetate.

Compound	Strain	Conc	Exposure 1 ΔOD at 24 hr	Exposure 2 ΔOD at 24 hr	Exposure 3 ΔOD at 24 hr
Potassium sorbate	101	0.1	0.26	0.16	
		0.2	0.26	0.20	0.19
		0.3	0.22	0.17	0.18
		0.4	0.16	0.15	0.18
		0.5	0.12	0.02	0.16
		0.6			0.05
	108	0.1	0.20	0.23	
		0.2	0.19	0.24	0.26
		0.3	0.13	0.12	0.23
		0.4	0.09	0.05	0.13
		0.5	0.05	0	0.04
		0.6			0
	310	0.1	0.24	0.19	
		0.2	0.20	0.19	0.19
		0.3	0.20	0.16	0.20
		0.4	0.20	0.15	0.19
		0.5	0.09	0.11	0.18
		0.6			0.18
	Scott A	0.1	0.20	0.2	
		0.2	0.20	0.21	0.21
		0.3	0.10	0.02	0.20
		0.4	0.10	0.01	0.04
		0.5	0.06	0	0.03
		0.6			0.01
Sodium benzoate	101	0.1	0.16	0.04	
		0.2	0.17	0.05	0.13
		0.3	0.16	0	0.07
		0.4	0.03	0	0.01
		0.5	0.02	0	0
		0.6			0
	108	0.1	0.13	0.08	
		0.2	0.15	0.10	0.16
		0.3	0.06	0.01	0.11
		0.4	0.01	0	0.01
		0.5	0.01	0	0
		0.6			0
	310	0.1	0.14	0.15	
		0.2	0.17	0.16	0.17
		0.3	0.11	0.05	0.17

Table 10.0 Continued

Compound	Strain	Conc	Exposure 1 ΔOD at 24 hr	Exposure 2 ΔOD at 24 hr	Exposure 3 ΔOD at 24 hr
		0.4	0.01	0	0.07
		0.5	0	0	0.01
		0.6			0
	Scott A	0.1	0.15	0.03	
		0.2	0.21	0.12	0.1
		0.3	0.09	0.03	0.15
		0.4	0.03	0	0.03
		0.5	0.02	0	0
		0.6			0
Sodium Lactate	101	1.0	0.24	0.85	
		2.0	0.68	0.83	
		3.0	0.67	0.81	
		4.0	0.78	0.87	
	108	1.0	0.21	0.36	
		2.0	0.26	0.19	
		3.0	0.26	0.20	
		4.0	0.26	0.21	
	310	1.0	0.45	0.49	
		2.0	0.17	0.10	
		3.0	0.16	0.09	
		4.0	0.20	0.09	
	Scott A	1.0	0.17	0.18	
		2.0	0.19	0.08	
		3.0	0.19	0.19	
		4.0	0.22	0.12	
Sodium Diacetate	101	0.5	0.55	0.34	
		1.0	0.25	0.25	
		2.0	0.27	0.21	
		3.0	0.12	0.16	
	108	0.5	0.39	0.21	
		1.0	0.04	0.12	
		2.0	0.07	0.12	
		3.0	0.01	0	
	310	0.5	0.38	0.11	
		1.0	0.02	0.05	
		2.0	0.02	0.07	
		3.0	0.01	0	
	Scott A	0.5	0.39	0.13	
		1.0	0.04	0.12	
		2.0	0.05	0.13	
		3.0	0.01	0.00	

Table 11.0 Differences in optical density (OD) for *Salmonella* Typhimurium at 24 hr after repeated to different antimicrobial concentrations of potassium sorbate, sodium benzoate, sodium lactate and sodium diacetate.

Compound	Strain	Conc	Exposure 1 ΔOD at 24 hr	Exposure 2 ΔOD at 24 hr	Exposure 3 ΔOD at 24 hr
Potassium sorbate	2380	0.1	0.13	0	
		0.2	0.12	0.01	0.02
		0.3	0.15	0	0.02
		0.4	0.19	0.04	0
		0.5	0	0	0.04
		0.6			0
	2486	0.1	0.05	0.01	
		0.2	0.06	0	0.03
		0.3	0	0.04	0.01
		0.4	0	0.01	0.06
		0.5	0	0	0
		0.6			0
	2576	0.1	0.09	0	
		0.2	0.10	0	.01
		0.3	0.08	0.01	.01
		0.4	0.04	0.02	0
		0.5	0	0	0.03
		0.6			0
	2582	0.1	0.10	0	
		0.2	0.11	0	0
		0.3	0.09	0	0
		0.4	0.04	0.03	0
		0.5	0	0	0.03
		0.6			0.03
Sodium benzoate	2380	0.1	0.02	0.04	
		0.2	0.08	0.02	0.08
		0.3	0	0.16	0
		0.4	0	0	0
		0.5	0	0	0
		0.6			0
	2486	0.1	0.01	0.02	
		0.2	0.05	0.04	0.03
		0.3	0	0.01	0.04
		0.4	0	0	0
		0.5	0	0	0.01
		0.6			0.01
	2576	0.1	0.02	-0.06	
		0.2	0.06	0.03	0.03
		0.3	0.01	0	0.04

Table 11.0 Continued

Compound	Strain	Conc	Exposure 1 ΔOD at 24 hr	Exposure 2 ΔOD at 24 hr	Exposure 3 ΔOD at 24 hr
		0.4	0.01	0	0
		0.5	0.01	0	0
		0.6			0.01
	2582	0.1	0.03	0.03	
		0.2	0.08	0.12	0.03
		0.3	0.01	0.01	0.01
		0.4	0	0.01	0
		0.5	0	0.01	0
		0.6			0
Sodium Lactate	2380	1.0	0.95	1.2	
		2.0	1.53	1.0	
		3.0	1.59	1.0	
		4.0	1.57	0.95	
	2486	1.0	0.56	1.35	
		2.0	1.38	1.14	
		3.0	1.43	1.15	
		4.0	1.38	1.17	
	2576	1.0	0.8	1.34	
		2.0	1.66	1.26	
		3.0	1.66	1.32	
		4.0	1.68	1.23	
	2582	1.0	0.8	1.38	
		2.0	1.66	1.23	
		3.0	1.66	1.26	
		4.0	1.68	1.14	
Sodium Diacetate	2380	0.5	0.50	1.11	
		1.0	0.02	0	
		2.0	0.02	0	
		3.0	0.01	0	
	2486	0.5	0.41	1.10	
		1.0	0.01	0.05	
		2.0	0.03	0.02	
		3.0	0	0	
	2576	0.5	0.34	0.77	
		1.0	0	0	
		2.0	0	0	
		3.0	0	0	
	2582	0.5	0.74	1.34	
		1.0	0.03	0.17	
		2.0	0.01	0.02	
		3.0	0	0	

was highly inhibitory at concentrations > 0.5% (Table 11, Appendix 21.0, 25.0, 29.0, 33.0). Since there was little or no growth in cell suspensions containing more than 1.0% sodium diacetate, it was not necessary to expose cells to a third higher concentration. At 0.5% however, transfer to the same concentration for 24 hr caused increased growth as demonstrated by an increased optical density for all strains.

IV. Discussion

Using an agar dilution assay, it was demonstrated that *Listeria monocytogenes* could be adapted to grow at increased concentrations of potassium sorbate, sodium benzoate, sodium lactate and sodium diacetate. The MIC of all strains of *L. monocytogenes* increased by > 0.2%, 0.1-0.2%, 2% and 0.5%, respectively, for the four compounds. Therefore, it could be concluded that cells could become adapted to increasing concentrations of the antimicrobial food preservatives. For *Salmonella* Typhimurium, similar results were seen. The MIC of all strains of *S.* Typhimurium 0.2 to > 0.3%, 0.1-0.2%, 2% and 0.5% for potassium sorbate, sodium benzoate, sodium lactate and sodium diacetate, respectively. A few other studies have reported that microorganisms may develop resistance to antimicrobial agents, such as weak organic acids upon subsequent exposure (Lueck, 1980; Brul and Coote, 1999; Lin et al., 1996; Davis et al., 2002).

The susceptibility of the two microorganisms was slightly different using a microbroth dilution assay compared to the agar dilution assay. For *L. monocytogenes*, resistance to potassium sorbate, was similar to that shown by the agar dilution assay. In contrast, the microorganism was appeared much less resistant to sodium benzoate and much more resistant to sodium lactate and sodium diacetate. For *S.* Typhimurium, potassium sorbate and sodium benzoate were much more effective in the microbroth assay than the agar dilution assay. As with *L. monocytogenes*, sodium lactate was less effective against *Salmonella* in the broth dilution. Only with sodium diacetate did the microorganism demonstrate similar susceptibility to that shown in the agar dilution assay. The primary reason for the differences was partly due to a difference in incubation time.

The agar diffusion assay was done for 48 hr while the dilution assay was done only for 24 hr. Incubation for 48 hr may have diminished the effect shown for those antimicrobials that demonstrated more activity in the agar dilution assay. Less effect in the broth dilution assay may have been due to exposure or contact with the microorganisms of the test compound. While there were differences in the antimicrobial effectiveness between the assays, the development of resistance or adaptation was demonstrated to a lesser extent in the dilution assay as well. *Listeria monocytogenes* strains 101 and 310 were shown to be able to grow at up to 0.6% potassium sorbate and strains 108 and Scott A grew better at 0.4 and 0.3%, respectively. *Salmonella* Typhimurium was generally able to adapt to sodium lactate at all concentrations and to sodium diacetate at lower concentrations.

The purpose of adjusting media pH to 6.0 was to prevent any pre-stress conditioning such as acid adaptation, from occurring that could contribute to the development of resistance to the food antimicrobials. It has been demonstrated that certain bacterial pathogens including *Salmonella*, *E. coli* and *Listeria monocytogenes*, when exposed to low pH (mildly acidic 5.5-6.0), may undergo changes that provide them with varying degrees of resistance to subsequent exposure to normally lethal acidic conditions (Davidson and Harrison, 2002). This increased resistance to low pH through pre-exposure has been termed "acid habituation", "acid tolerance" or "acid shock". For example, studies reported *Salmonella* serovars Typhimurium, Enteritidis, Heidelberg and Javiana cells that were pre-exposed to a mild pH 5.8 showed increased resistance to food antimicrobials such as lactic, propionic and acetic acid (Leyer and Johnson, 1992). Others

like Gahan et al., (1996) demonstrated that by adapting *L. monocytogenes* LO28 to lactic acid at pH 5.5 for 60 min, cells had enhanced survival in low acid foods such as yogurt, cottage cheese, orange juice and salad dressings. Many of these studies have found that increased resistance was dependent upon the strain and environmental conditions (i.e. pH, growth medium). In the present study, results indicate that without any previous stress or acid adaptation, cells were able to adapt to higher antimicrobial concentrations.

Adaptation to the four antimicrobials persisted for both microorganisms for several weeks at 4°C. Strains did not lose viability and showed the similarly or only slightly less susceptibility to the previously adapted concentrations. One attribute that may have contributed to the survival of *Listeria monocytogenes* is the fact that it is a psychrotroph and can thus grow at refrigeration temperatures even under stress (Hill and Gahan, 2000). Lu et al., (2005) showed that when they reported that *L. monocytogenes* survived in refrigerated storage in the presence of 6.0% sodium diacetate.

Resistance by *Listeria monocytogenes* and *Salmonella* Typhimurium cells to any type of antimicrobial compound may be mediated by multiple mechanisms and/or resistance determinants in the bacterial cell. Some cells will grow and survive after antimicrobial exposure because they may possess a degree of natural or innate resistance (i.e., altered permeability, efflux pumps), or may acquire it latter through mutation or genetic exchange (Bower and Daeschel, 1999). In food application of antimicrobials, innate resistance may be influenced by environment, food component interactions, processing interactions or presence of antagonistic inhibitors (Davidson and Harrison, 2003). Acquired resistance is of greatest concern for use of food preservative antimicrobials.

While acquired resistance to antimicrobials is rare (Russell et al., 1997) investigations into the potential for such resistance are of extreme importance to the future use of traditional food antimicrobials (Davidson and Harrison, 2003). Food antimicrobials should not contribute to the development of resistant strains nor alter the environment of the food in such a way that growth of another pathogen is selected (Davidson and Branen, 2005). When microorganisms sense a stress, cells respond by activating an adaptive or stress response that increases tolerance to the same or to a different stress (Yousef and Courtney, 2003). It has been suggested that that the direct use of certain food antimicrobials may impose a selective pressure and contribute to the emergence of resistant microorganism in food environments (Russell, 2000). In the food-processing environment, bacteria are exposed to multiple stresses (e.g., heat, antimicrobials compounds), which are mainly used to preserve quality, increase shelf life and improve safety. These sublethal preservation stresses may result in cells that are less susceptible to subsequent stresses (Ravishankar and Juneja, 2003). Therefore, bacteria present in the plant environment survive and may be able to adapt to even harsher treatments (Ravishankar and Juneja, 2003).

In this study, the susceptibility of *Listeria monocytogenes,* a Gram positive bacterium, and *S.* Typhimurium, a Gram negative bacterium, could be compared. Depending on the assay and the antimicrobial compound, the developed resistance was variable. Adaptive resistance of the two types of microorganisms is most likely related to mechanisms involving destruction or inactivation of toxic compounds, changes in the target site, or active efflux of the chemicals out of the cell as well as changes in the outer

membrane of Gram negatives or cytoplasmic membrane of both (Russell, 2001). Finally, it was demonstrated that a multiple antibiotic-resistant pathogen, such as *Salmonella* Typhimurium DT104, was not necessarily more resistant to regulatory approved food antimicrobials than *Listeria monocytogenes*.

Actual exposure of foodborne pathogens in the food industry could be expected to be similar to the model used in this study. That is, if pathogenic microorganisms were re-exposed to an antimicrobial food preservative, it would be repeated exposure to the same or similar concentrations. Under these conditions, we found some evidence of increased resistance to traditional regulatory-approved food antimicrobials when exposed to repeated low concentrations. In addition, these cells were not acid adapted indicating that, without such adaptation; there was some level of resistance and adaptation. It is important to note that adapted or tolerant microorganisms would have to possess enhanced survival in order to survive in a food system. Again, this will depend on the physiological status of the organism, the type and concentration of antimicrobial and the physicochemical characteristics of the external environment.

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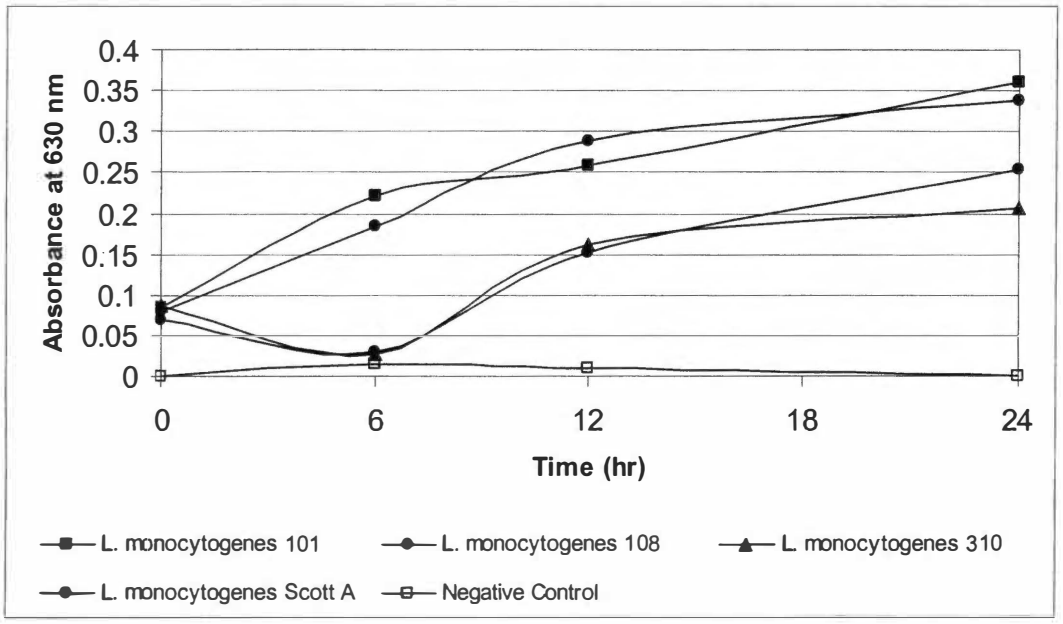
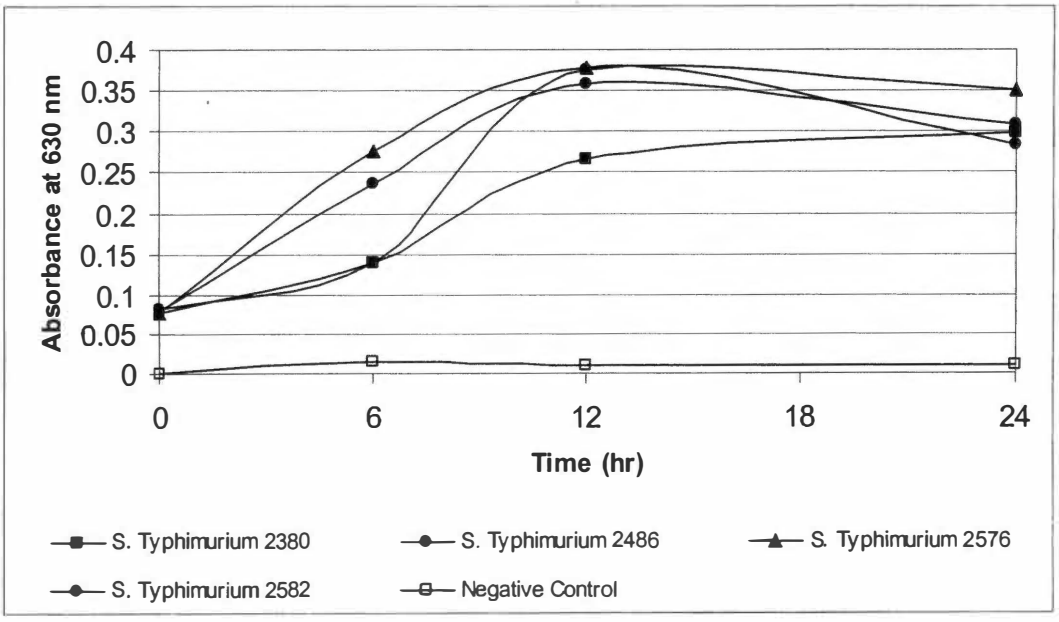
APPENDIX

Appendix 1.0: Growth of parent strains for *L. monocytogenes* (101, 108, 310, Scott A) (A) and *S.* Typhimurium (2380, 2486, 2576, 2582) (B) at pH 6.0 in 24 h.

A.

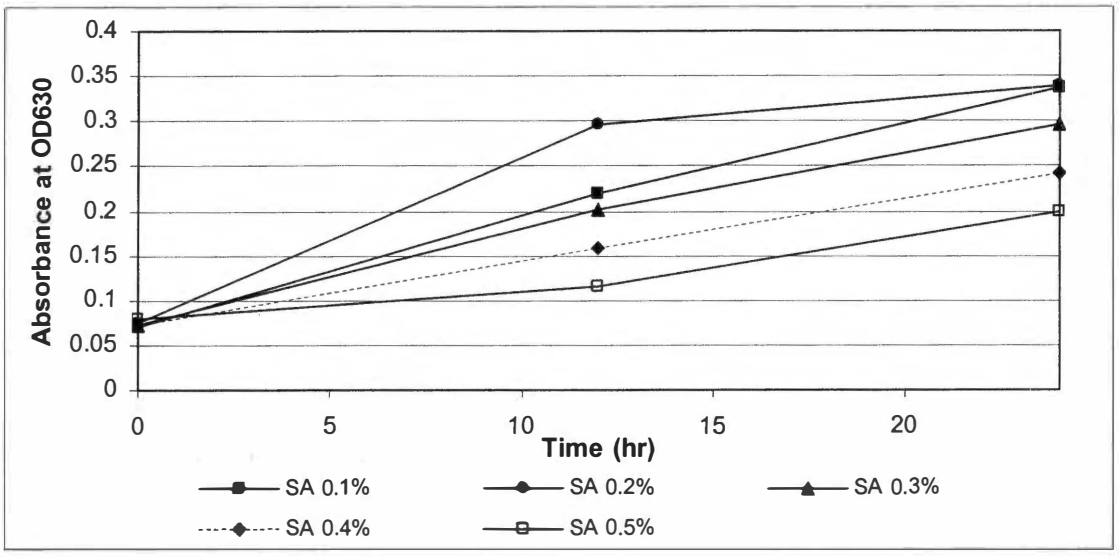


B.

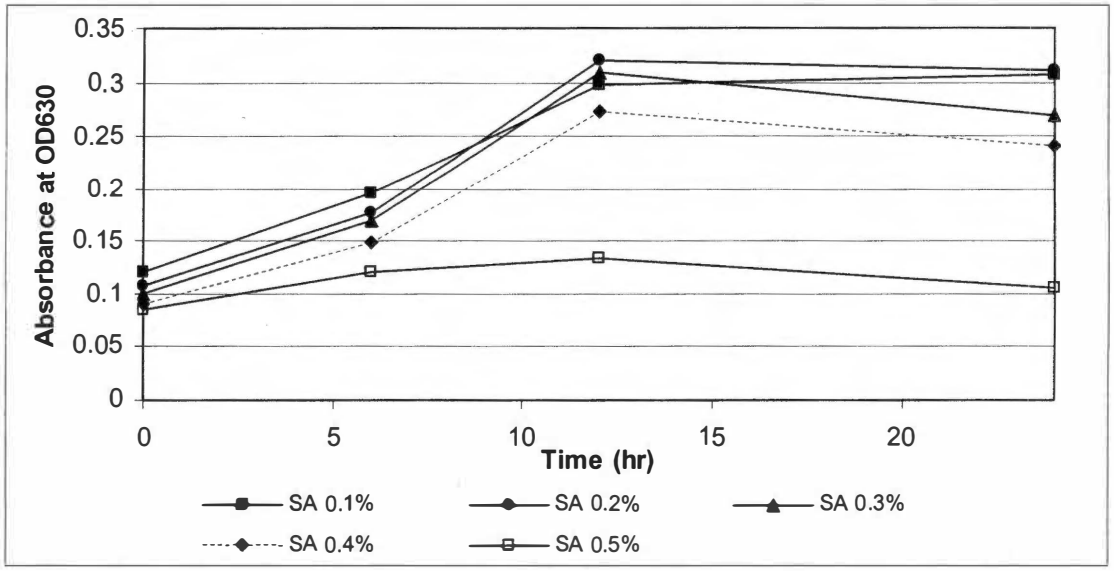


Appendix 2.0: Exposure of *L. monocytogenes* strain 101 to potassium sorbate (PS) at different concentrations at pH 6.0 in 24 h. A) Initial exposure B) Second exposure of cells at same initial concentration. C) Third exposure at one step higher concentration.

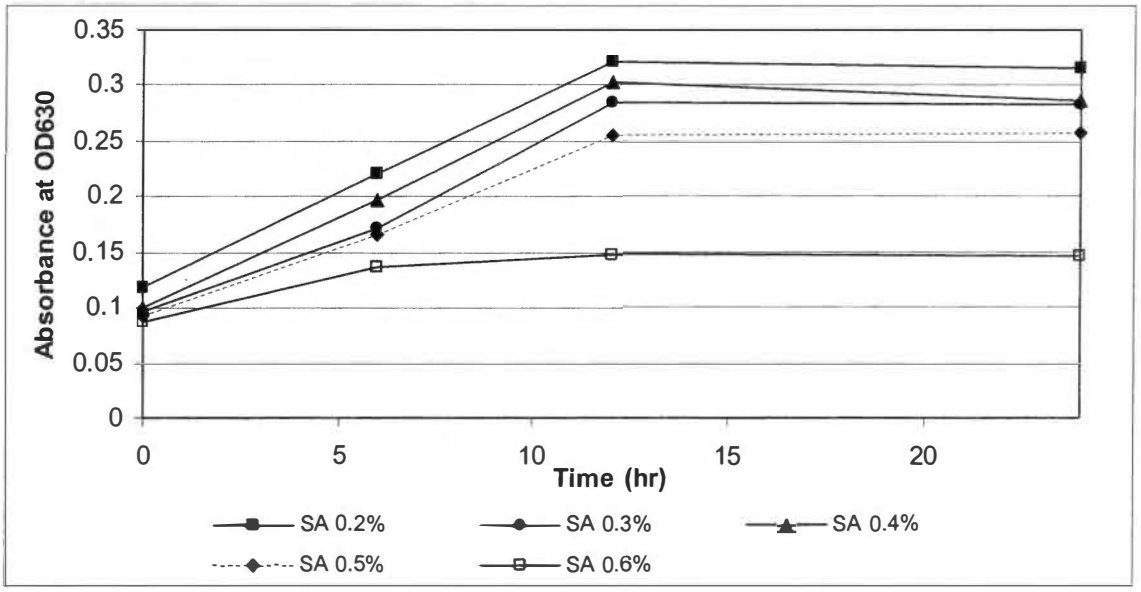
A.



B.

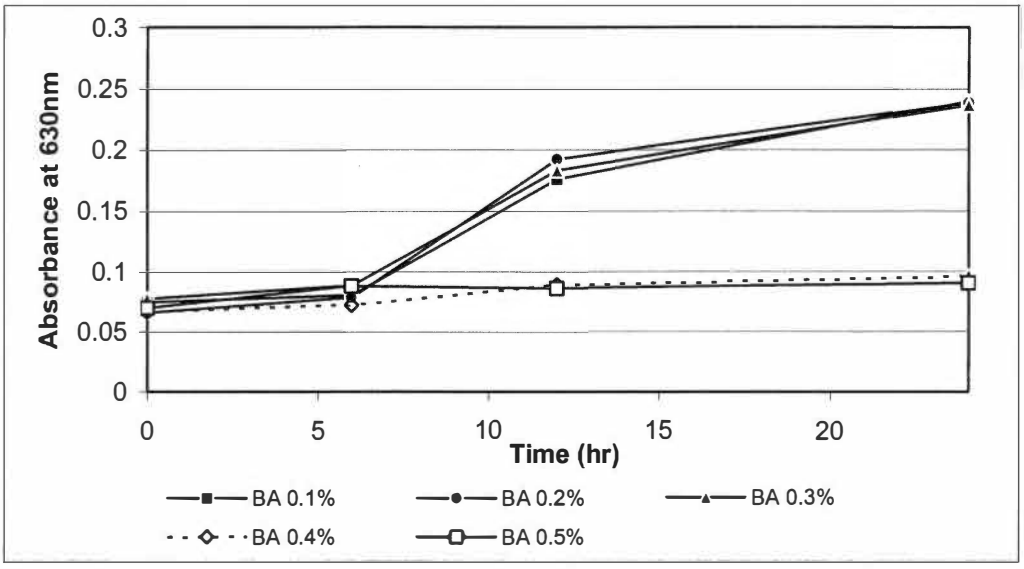


C.

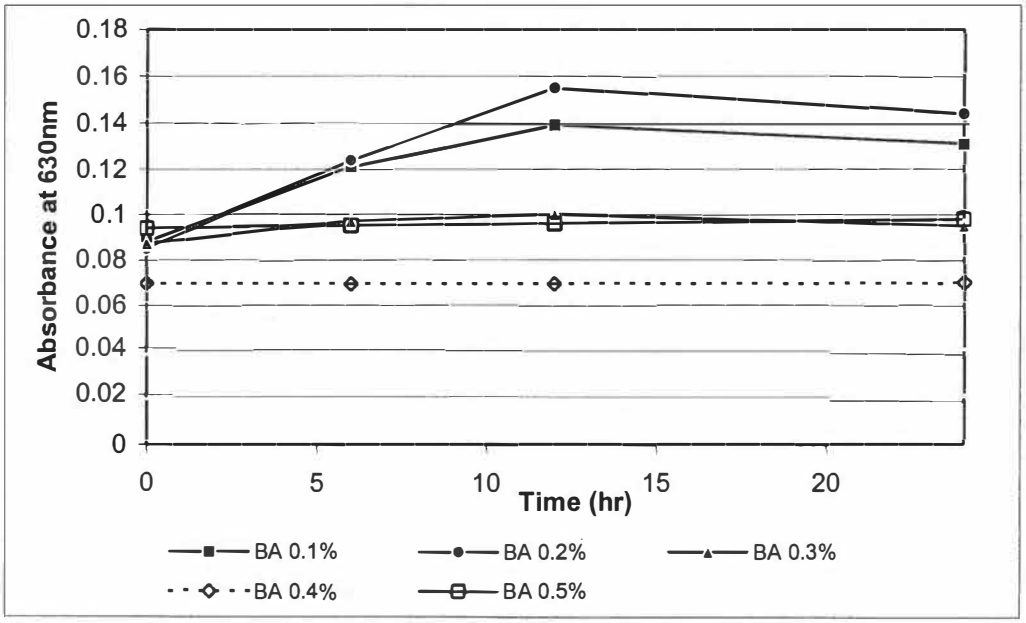

Absorbance at OD630
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
Time (hr)
SA 0.2%
SA 0.3%
SA 0.4%
SA 0.5%
SA 0.6%

Appendix 3.0: Exposure of *L. monocytogenes* strain 101 to benzoic acid (BA) at different concentrations at pH 6.0 in 24 h. (A) Initial exposure B) Second exposure of cells at same initial concentration C) Third exposure at one step higher concentration.

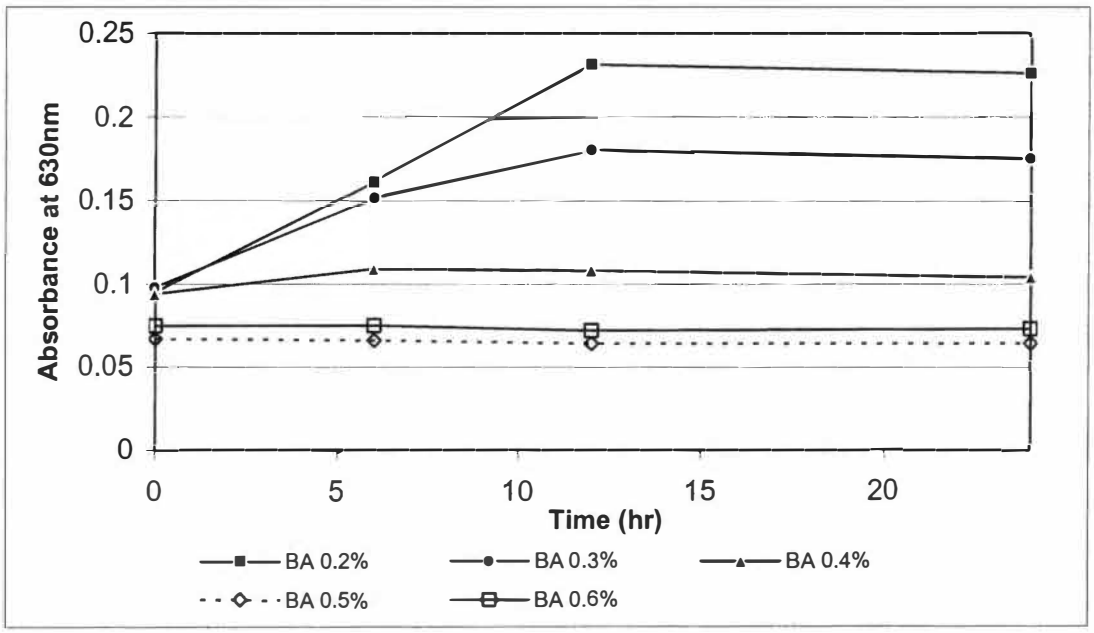
A.



B.

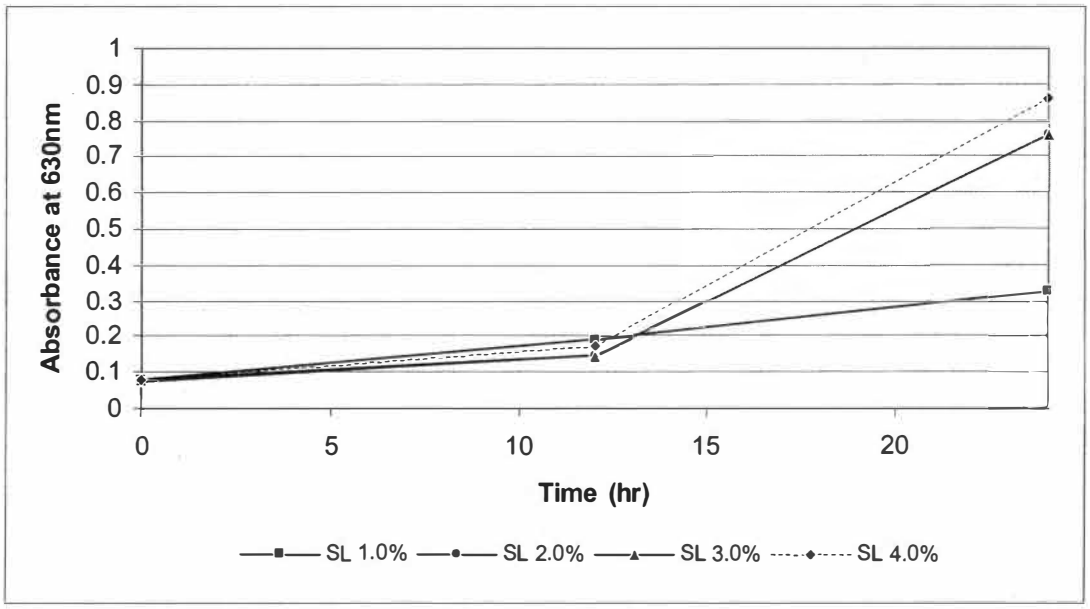


C.

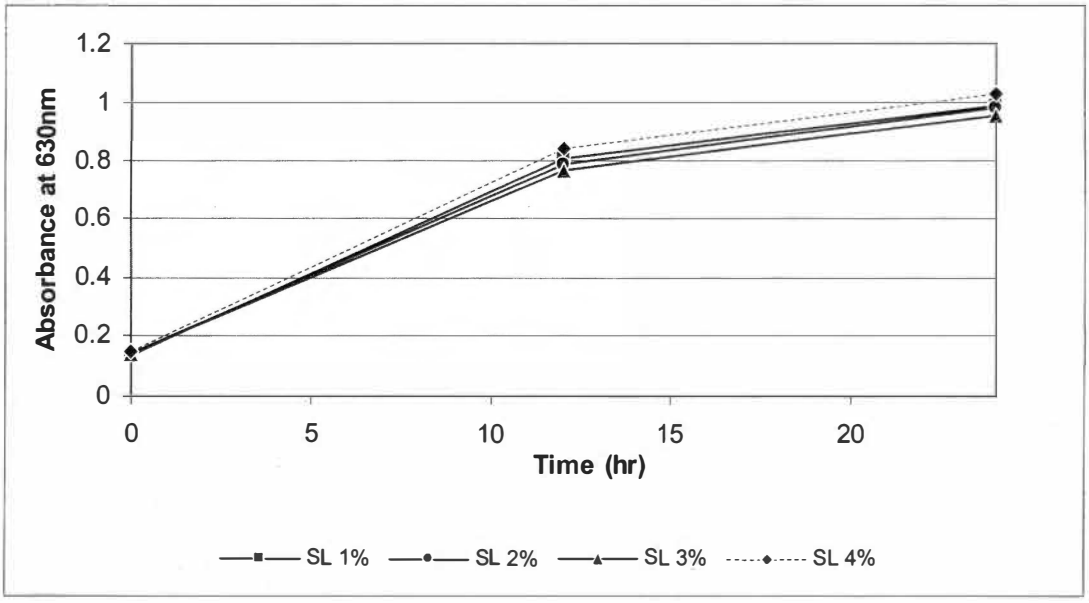

0.25
0.2
0.15
0.1
0.05
0
Absorbance at 630nm
0
5
10
15
20
Time (hr)
BA 0.2%
BA 0.3%
BA 0.4%
BA 0.5%
BA 0.6%

Appendix 4.0: Exposure of *L. monocytogenes* strain 101 to sodium lactate (SL) at different concentrations at pH 6.0 in 24 h.
A) Initial exposure B) Second exposure of cells at same initial concentration.

A.

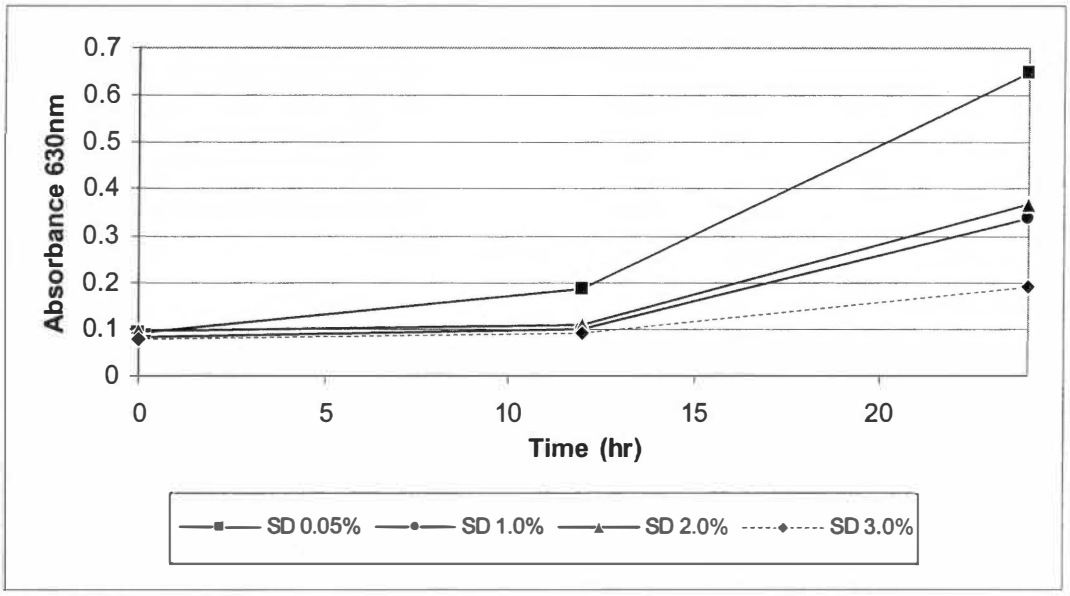


B.

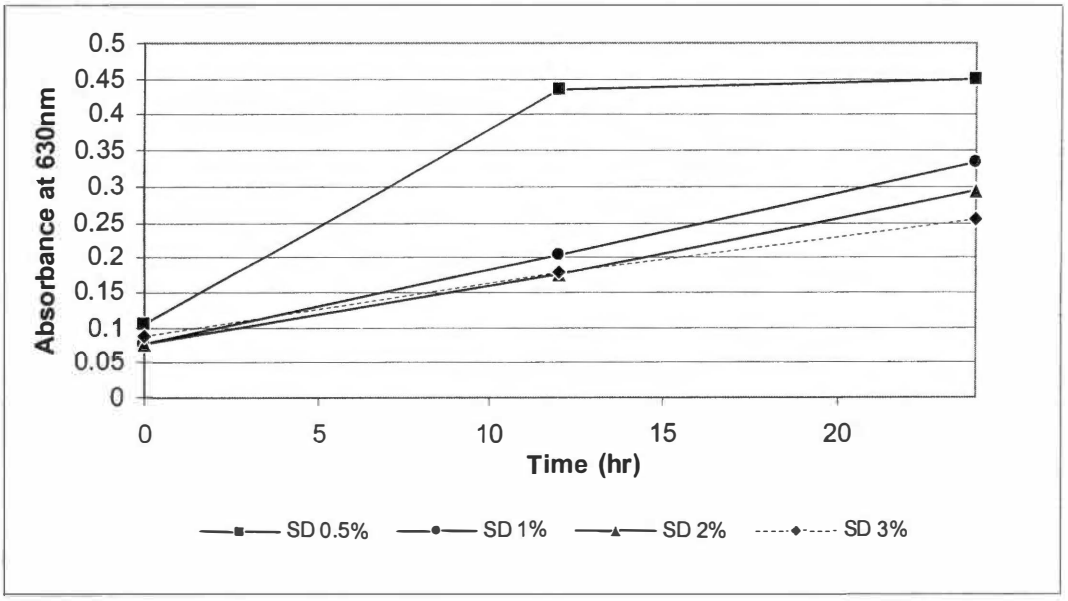


Appendix 5.0: Exposure of *L. monocytogenes* strain 101 to sodium diacetate (SD) at different concentration at pH 6.0 in 24 h.
A) First exposure B) Second exposure of cells at same initial concentration.

A.

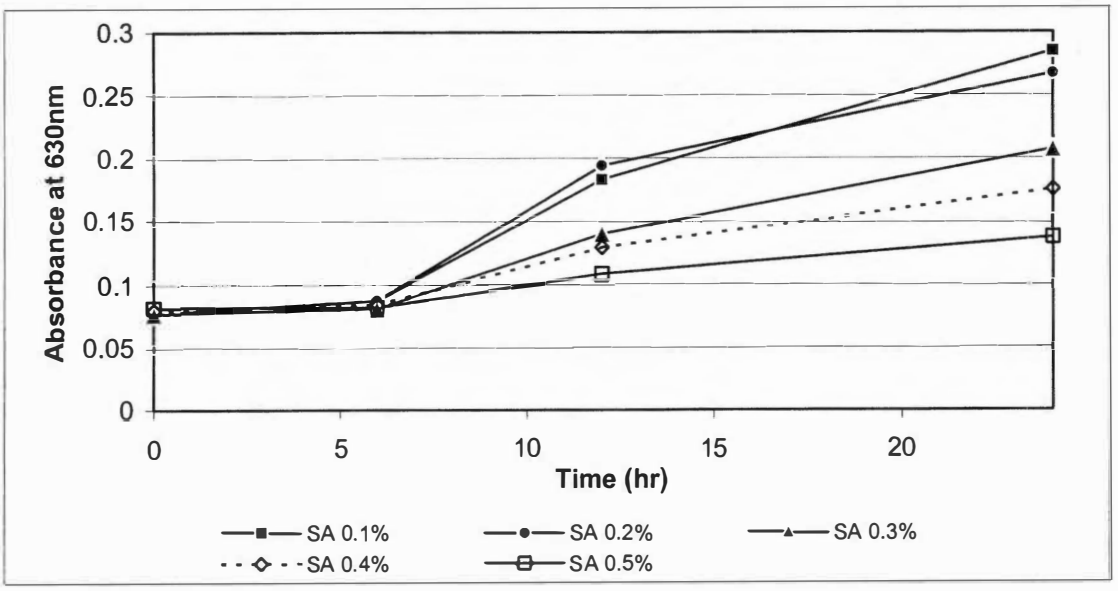


B.

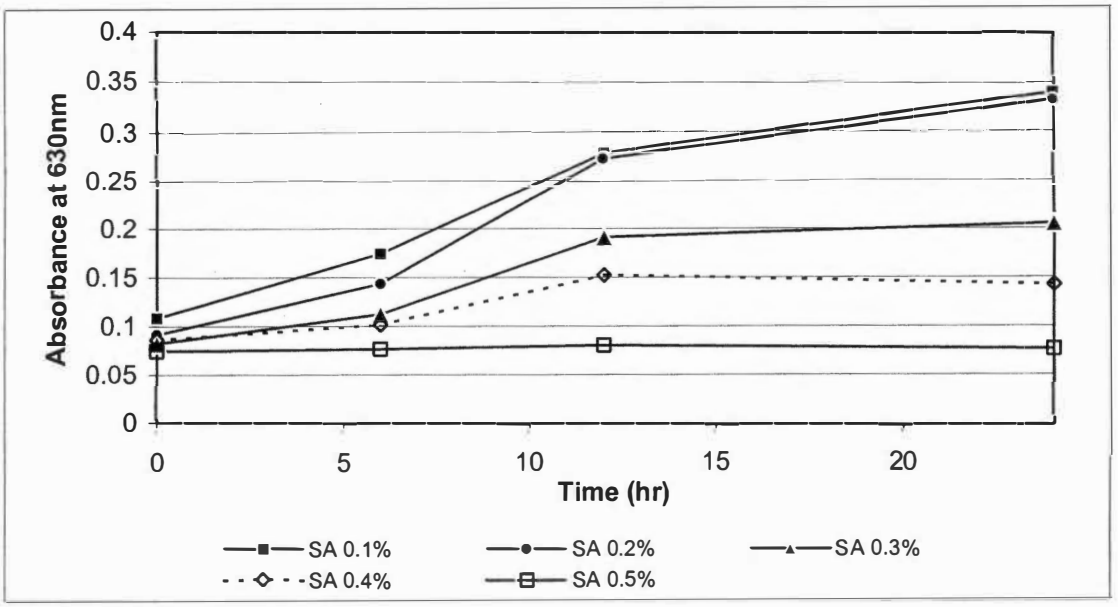


Appendix 6.0: Exposure of *L. monocytogenes* strain 108 to sorbic acid (SA) at different concentrations at pH 6.0 in 24 h. A) Initial exposure B) Second exposure of cells at same initial concentration. C) Third exposure at one step higher concentration.

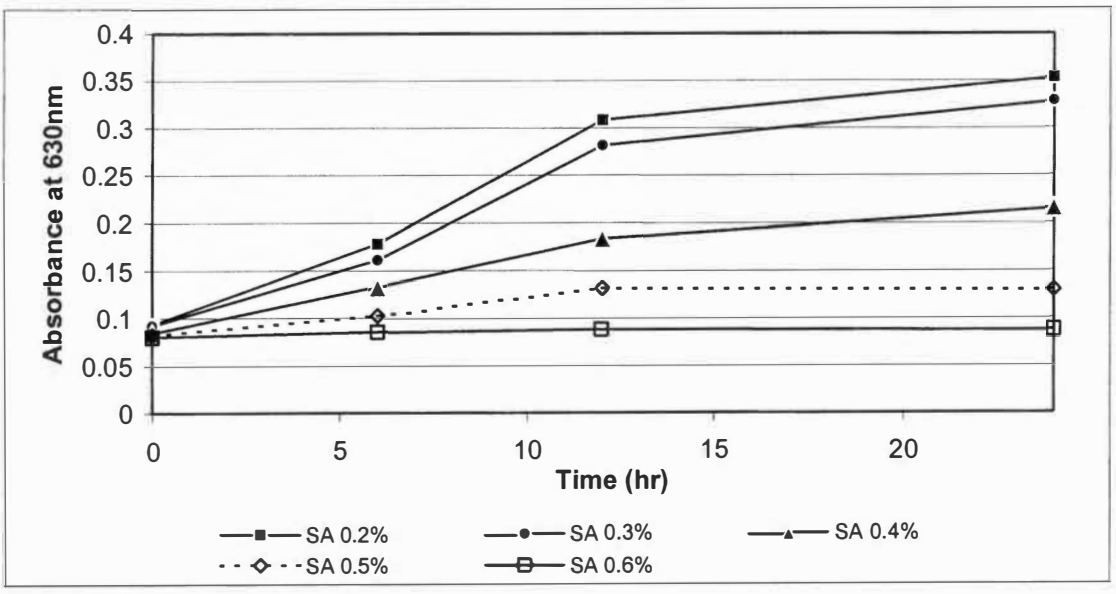
A.



B.

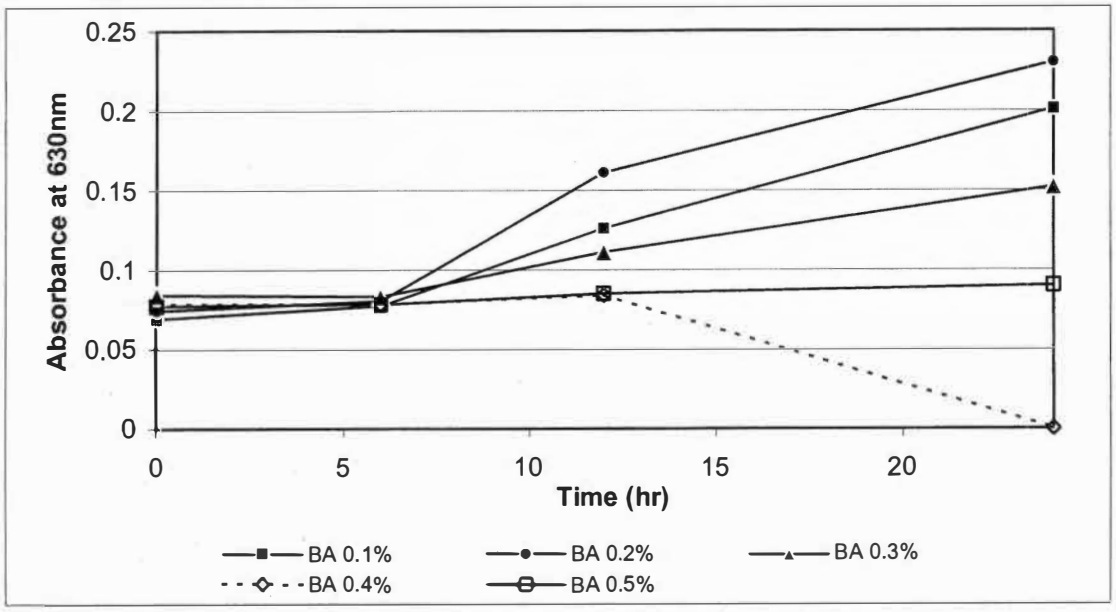


C.

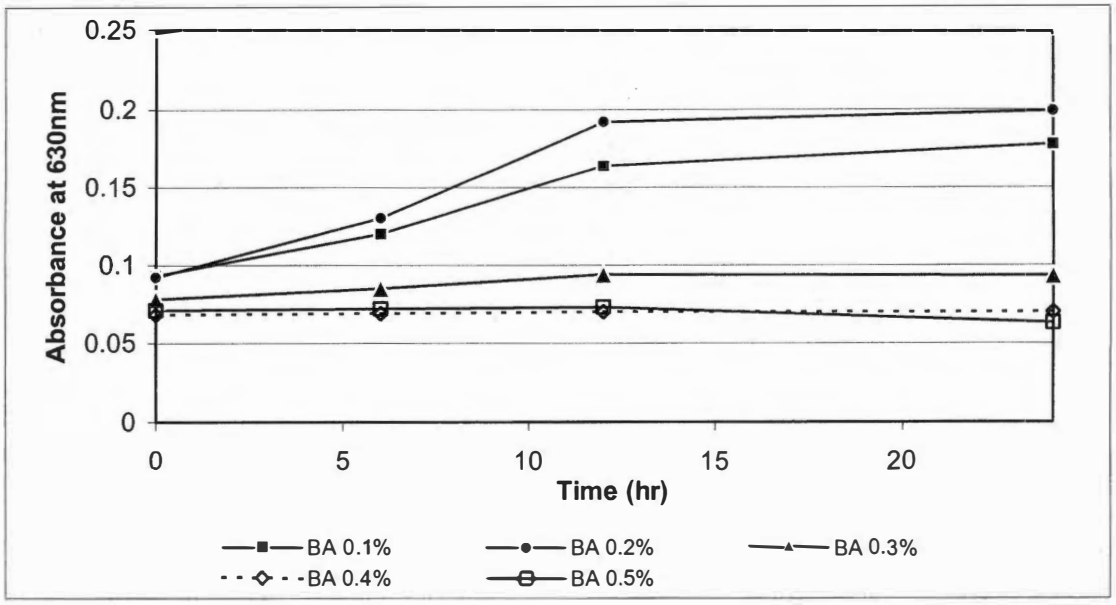

Absorbance at 630nm
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
Time (hr)
SA 0.2%
SA 0.3%
SA 0.4%
SA 0.5%
SA 0.6%

Appendix 7.0: Exposure of *L. monocytogenes* strain 108 to benzoic acid (BA) at different concentrations at pH 6.0 in 24 h. A) Initial exposure B) Second exposure of cells at same initial concentration. C) Third exposure at one step higher concentration.

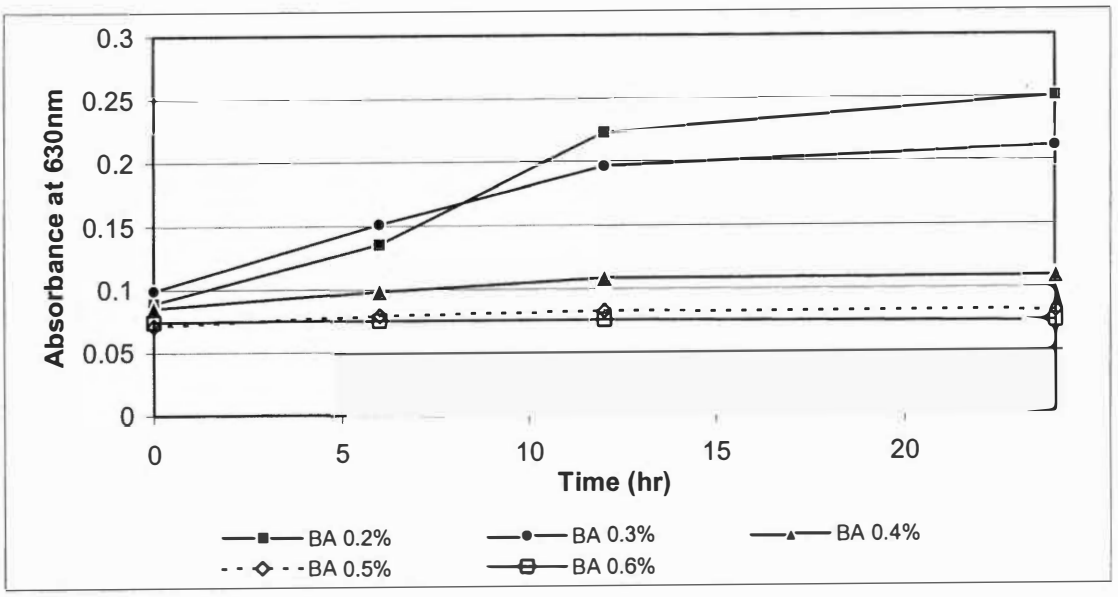
A.



B.

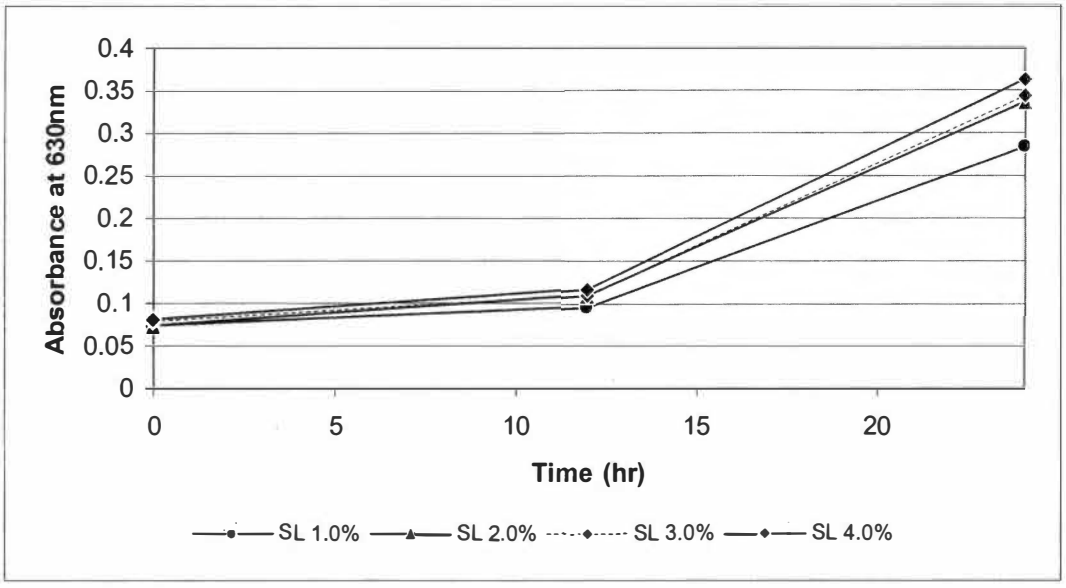


C.

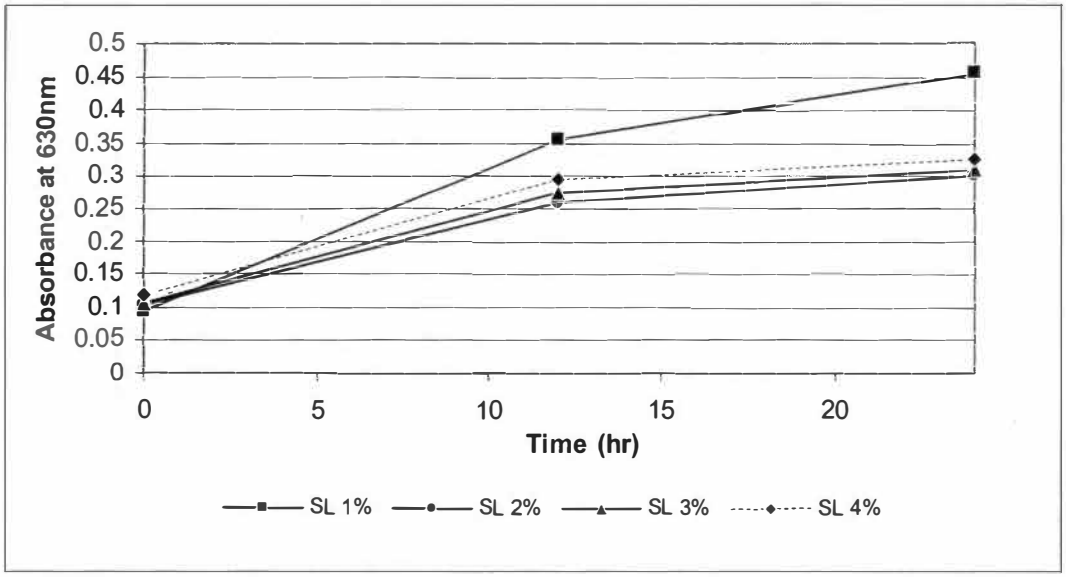

Absorbance at 630nm
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
Time (hr)
BA 0.2%
BA 0.3%
BA 0.4%
BA 0.5%
BA 0.6%

Appendix 8.0: Exposure of *L. monocytogenes* strain 108 to sodium lactate (SL) at different concentration at pH 6.0 in 24 h.
A) Initial exposure B) Second exposure of cells at same initial concentration.

A.

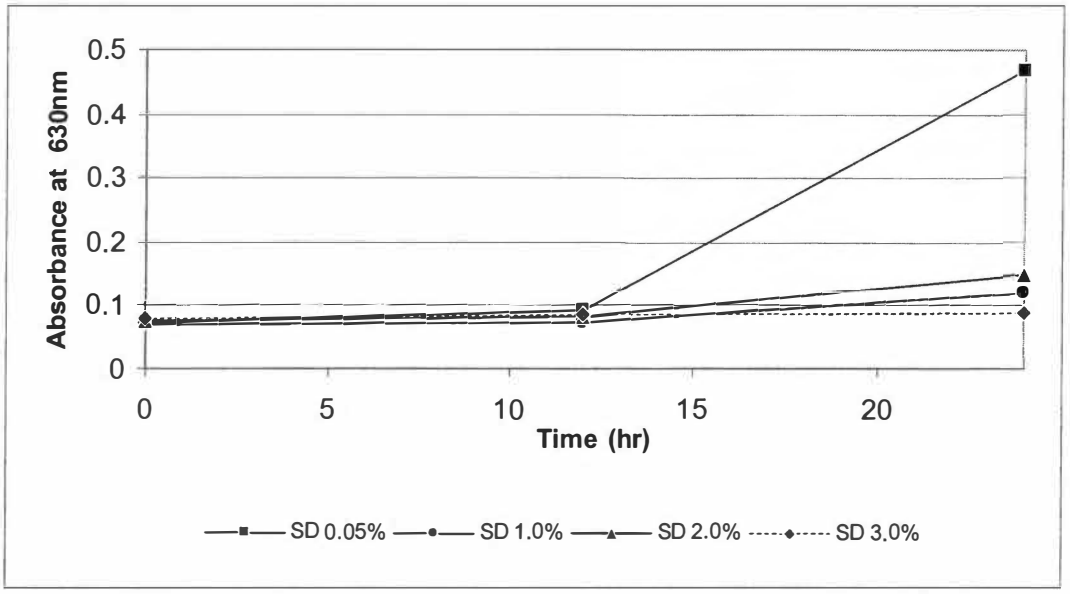


B.

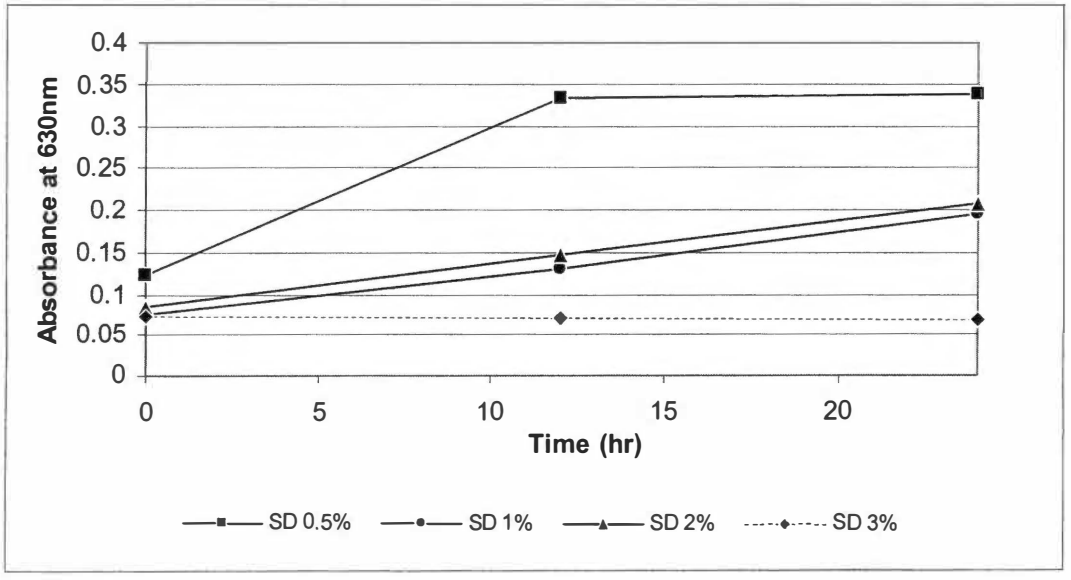


Appendix 9.0: Exposure of *L. monocytogenes* strain 108 to sodium diacetate (SD) at different concentration at pH 6.0 in 24 h.
A) First exposure B) Second exposure of cells at same initial concentration.

A.

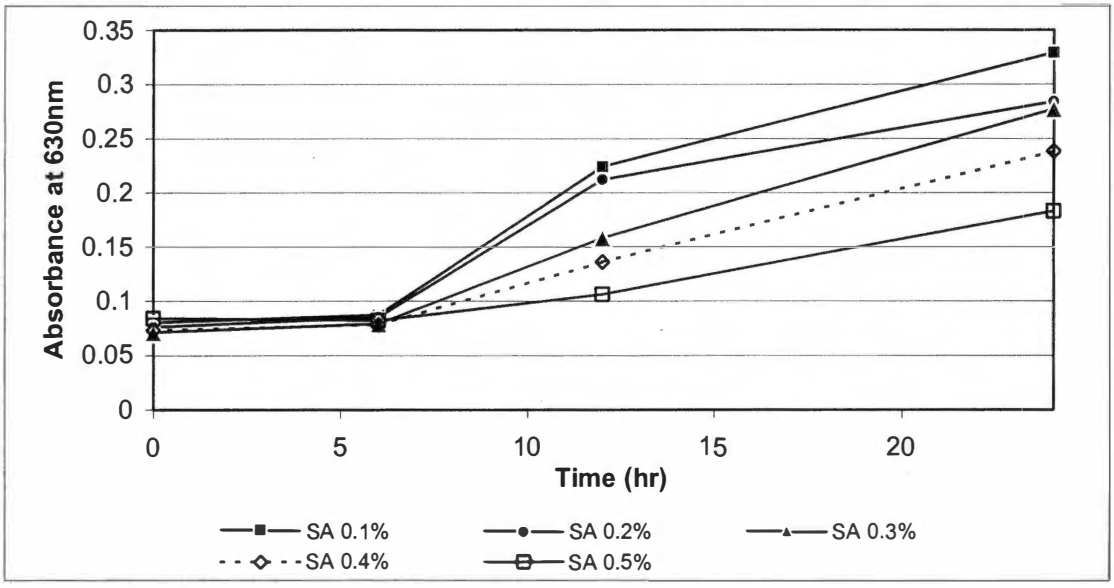


B.

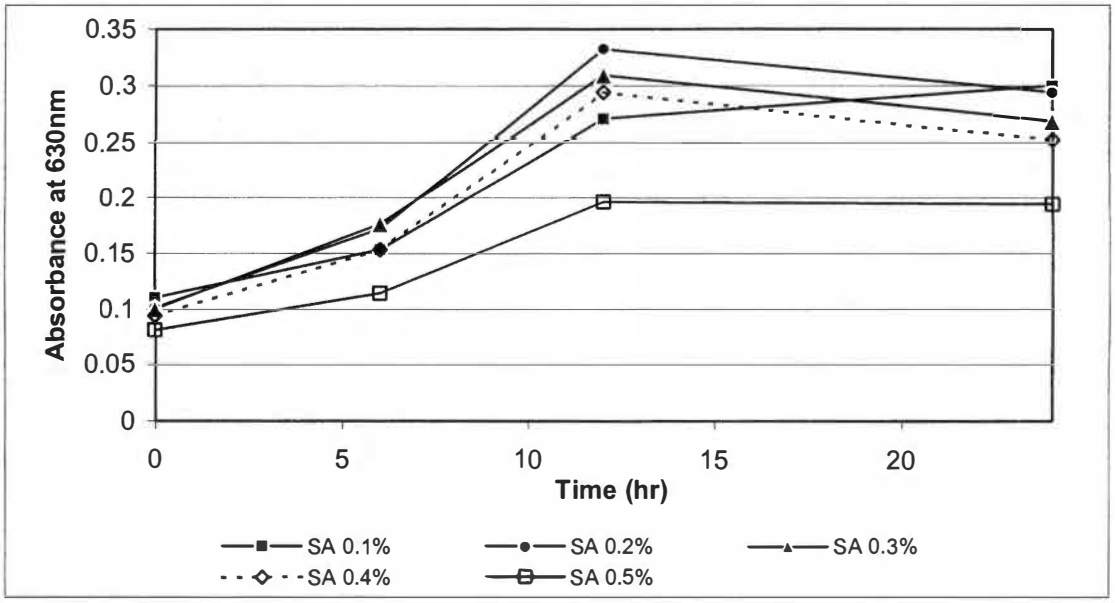


Appendix 10.0: Exposure of *L. monocytogenes* strain 310 to sorbic acid (SA) at different concentrations at pH 6.0 in 24 h. A) Initial exposure B) Second exposure of cells at same initial concentration. C) Third exposure at one step higher concentration.

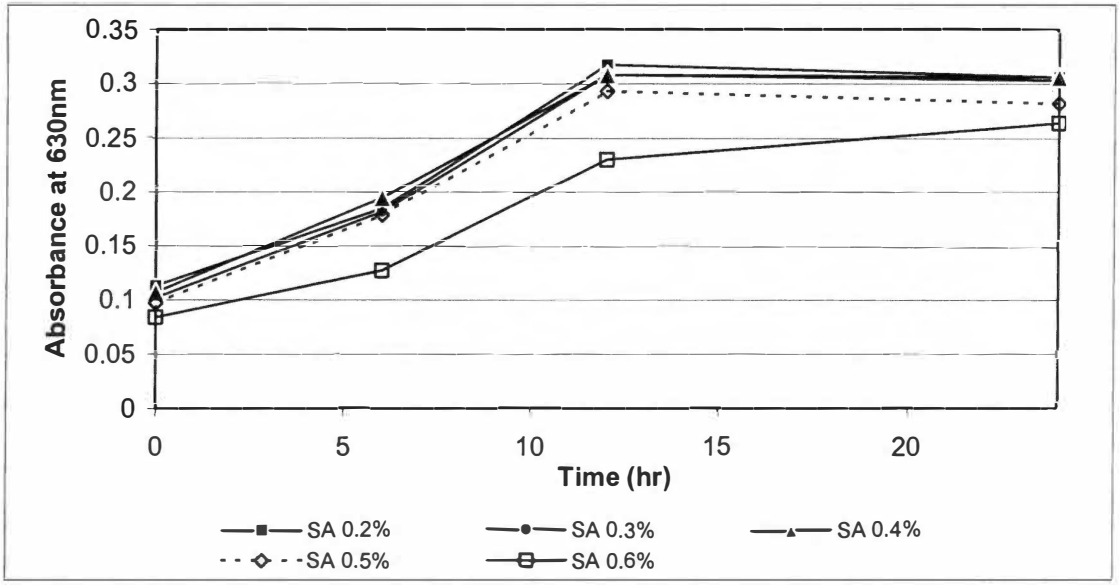
A.



B.

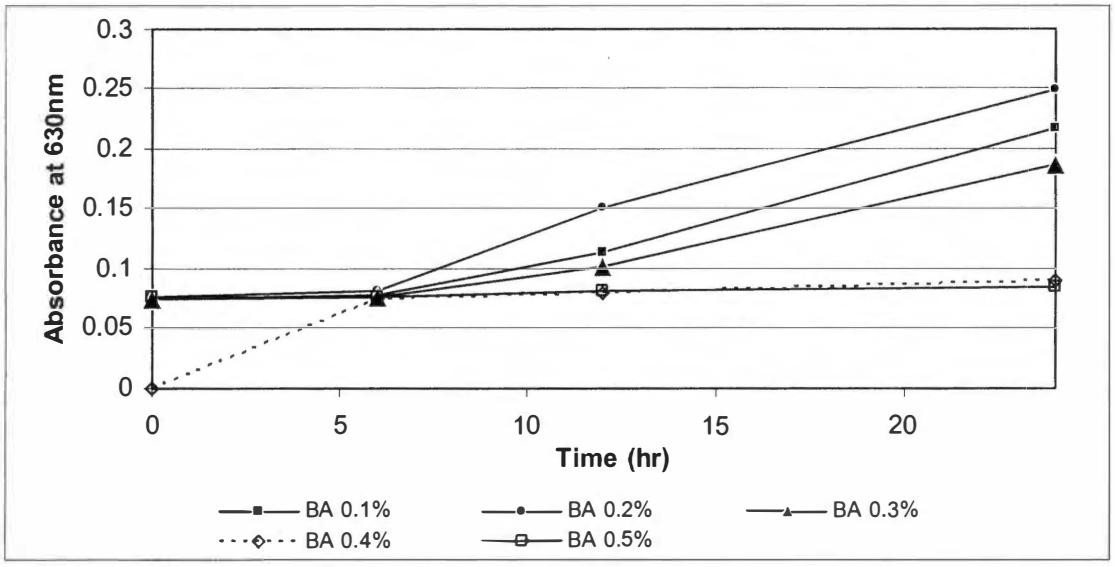


C.

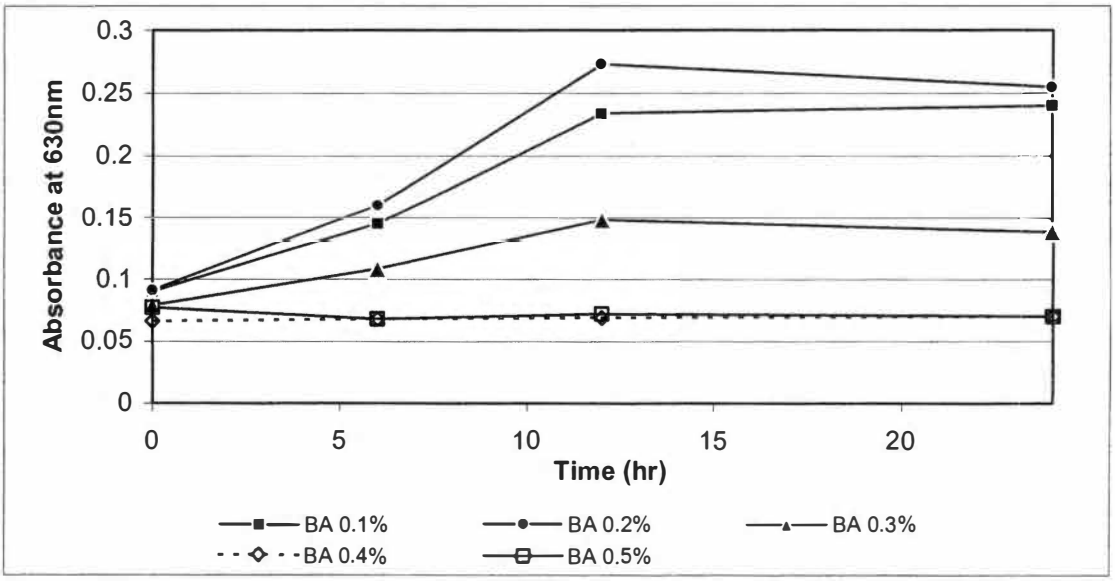

Absorbance at 630nm
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
Time (hr)
SA 0.2%
SA 0.3%
SA 0.4%
SA 0.5%
SA 0.6%

Appendix 11.0: Exposure of *L. monocytogenes* strain 310 to benzoic acid (BA) at different concentrations at pH 6.0 in 24 h. A) Initial exposure B) Second exposure of cells at same initial concentration. C) Third exposure at one step higher concentration.

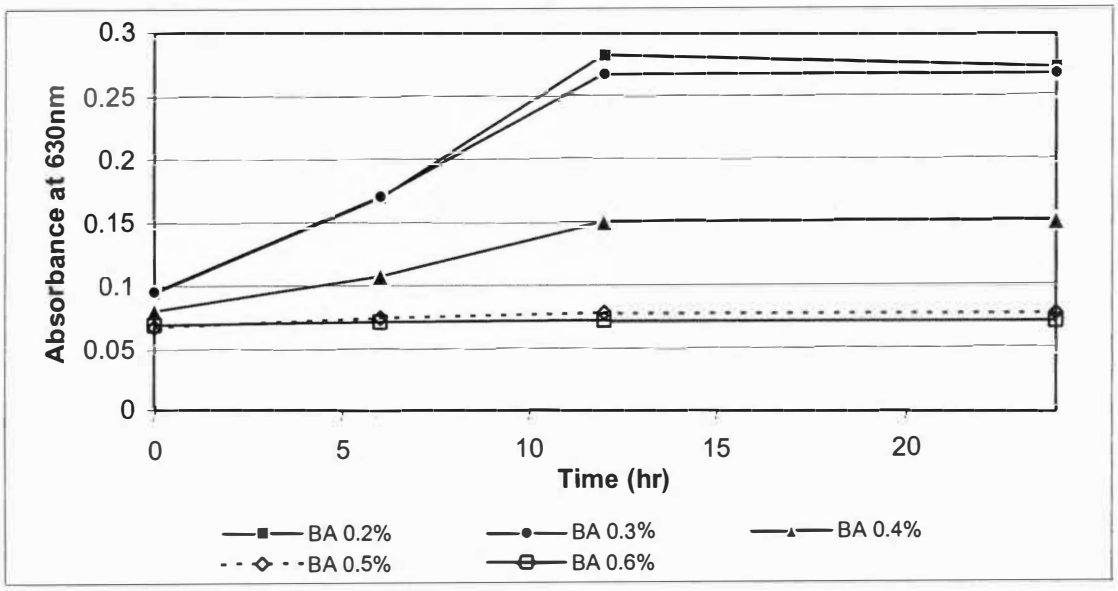
A.



B.

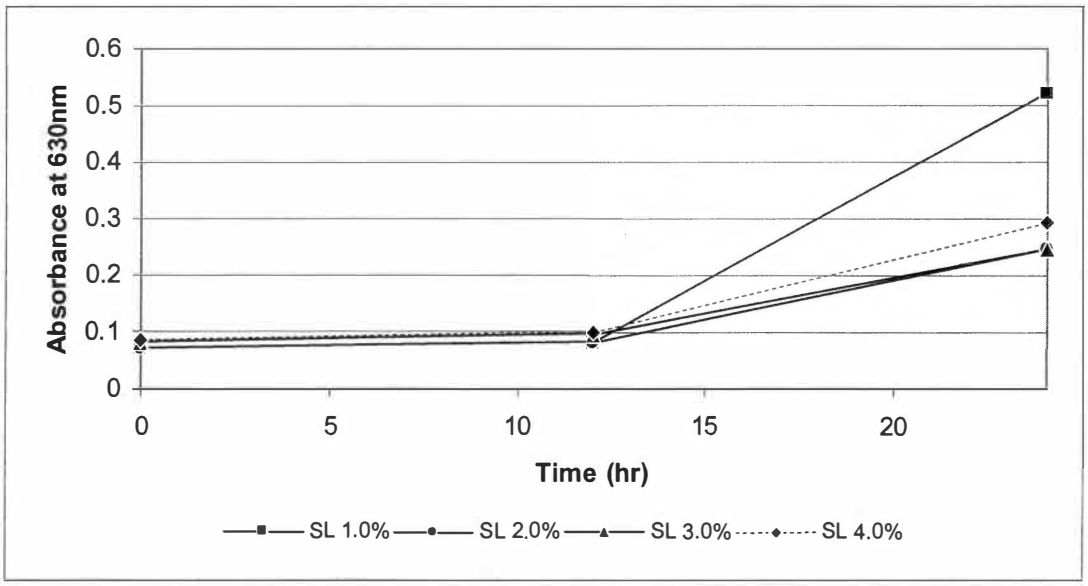


C.

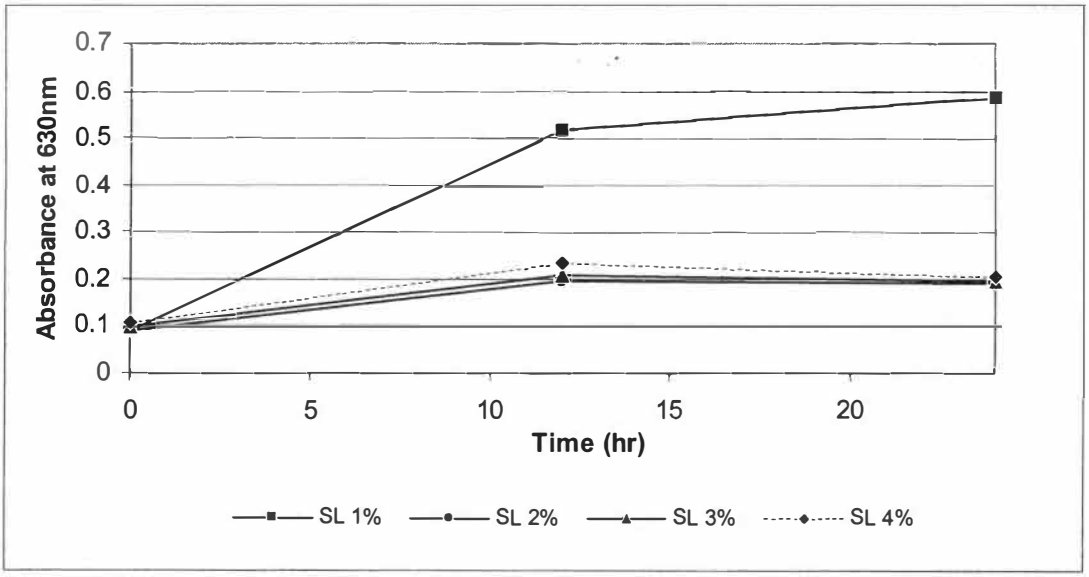

Absorbance at 630nm
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
Time (hr)
BA 0.2%
BA 0.3%
BA 0.4%
BA 0.5%
BA 0.6%

Appendix 12.0: Exposure of *L. monocytogenes* strain 310 to sodium lactate (SL) at different concentration at pH 6.0 in 24 h.
A) First exposure B) Second exposure of cells at same initial concentration.

A.

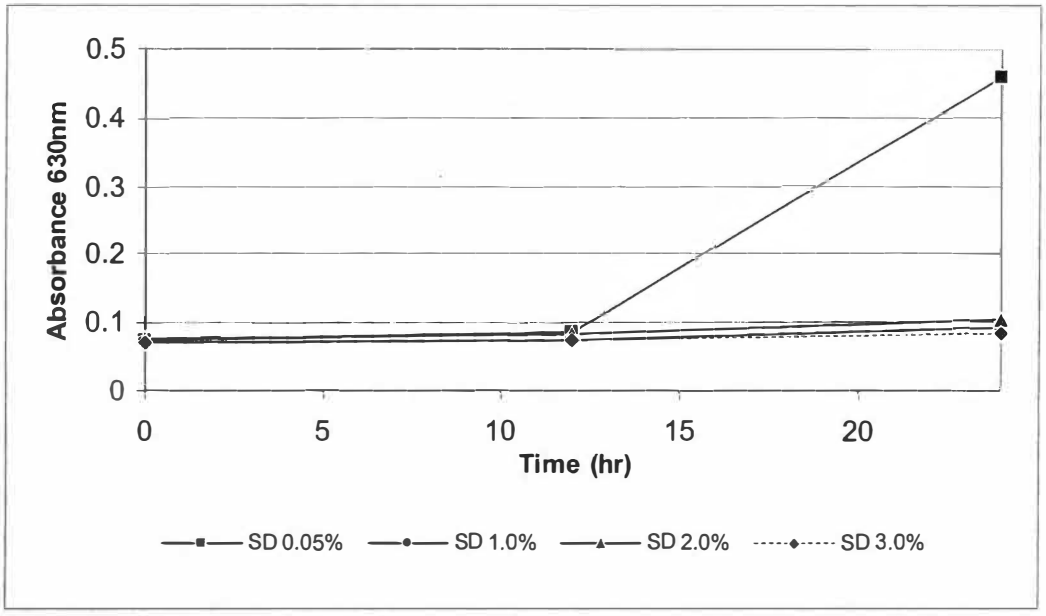


B.

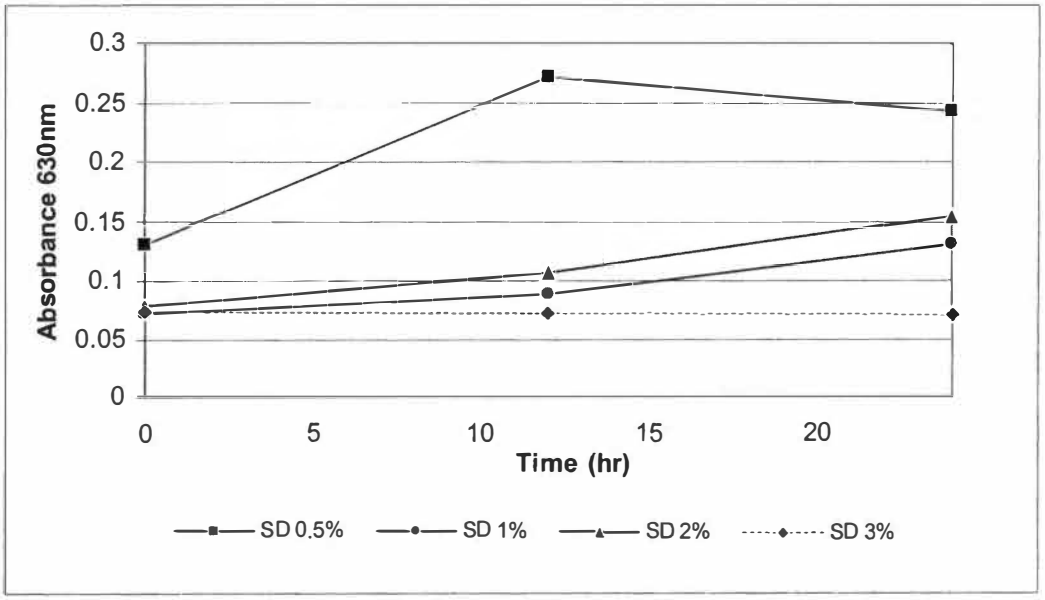


Appendix 13.0: Exposure of *L. monocytogenes* strain 310 to sodium diacetate (SD) at different concentration at pH 6.0 in 24 h.
A) First exposure B) Second exposure of cells at same initial concentration.

A.

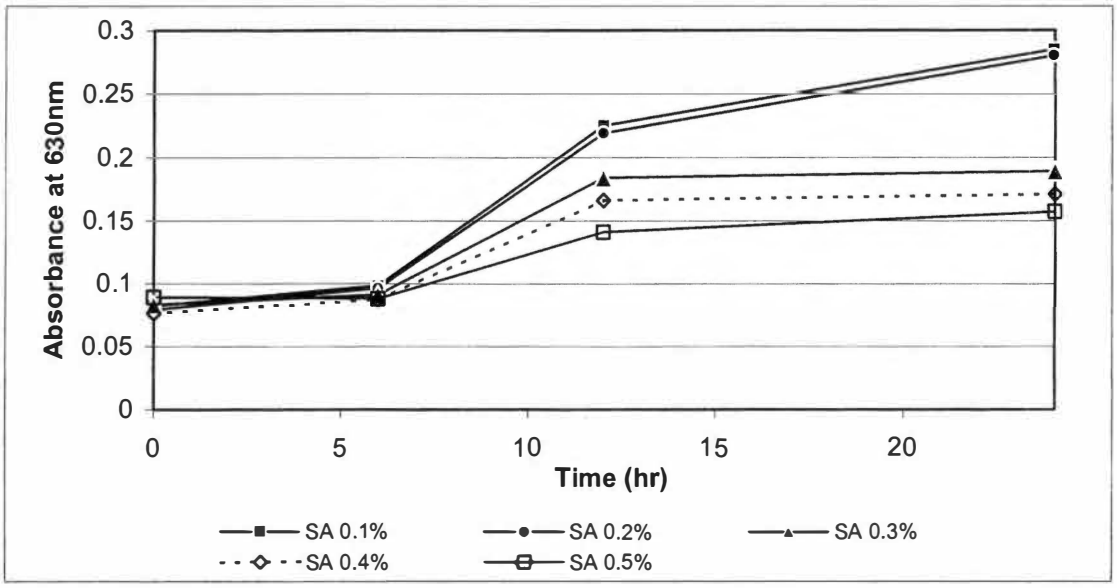


B.

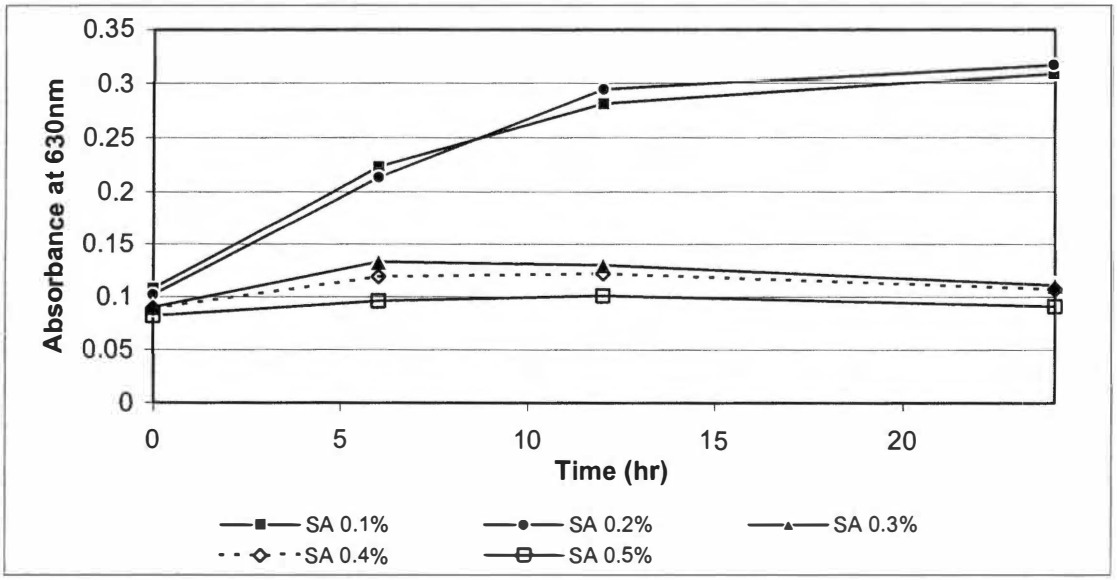


Appendix 14.0: Exposure of *L. monocytogenes* strain Scott A to sorbic acid (SA) at different concentrations at pH 6.0 in 24 h. A) Initial exposure B) Second exposure of cells at same initial concentration. C) Third exposure at one step higher concentration.

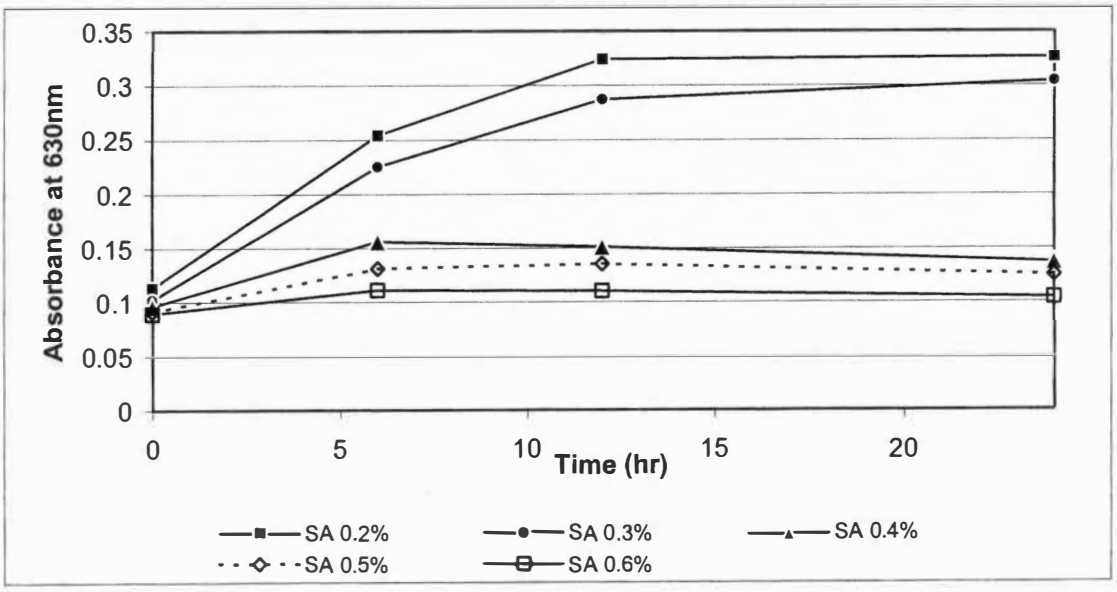
A.



B.

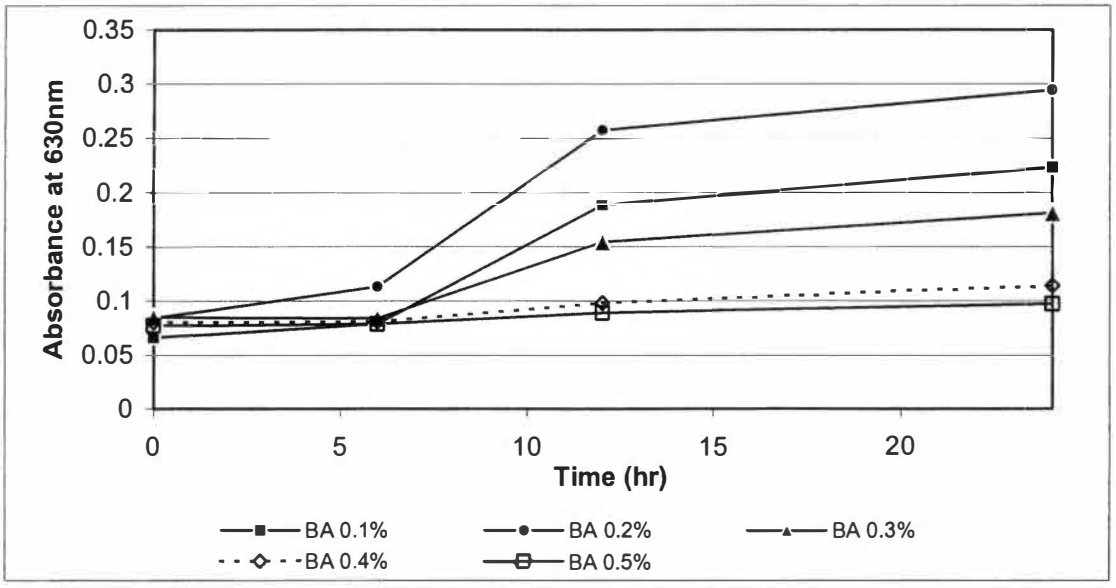


C.

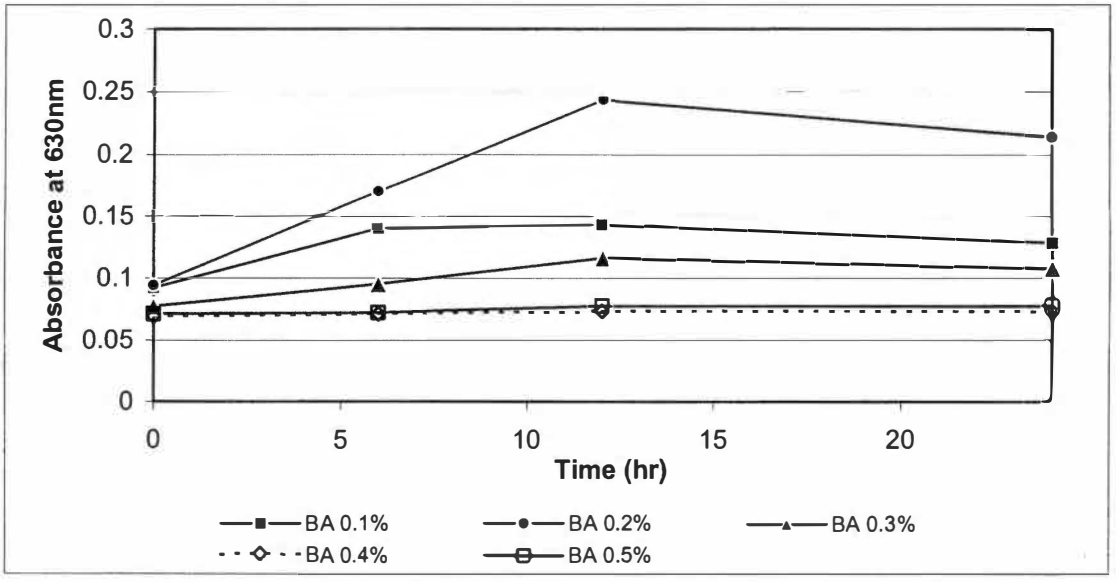

Absorbance at 630nm
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
Time (hr)
SA 0.2%
SA 0.3%
SA 0.4%
SA 0.5%
SA 0.6%

Appendix 15.0: Exposure of *L. monocytogenes* strain Scott A to benzoic acid (BA) at different concentrations at pH 6.0 in 24 h. A) Initial exposure B) Second exposure of cells at same initial concentration. C) Third exposure at one step higher concentration.

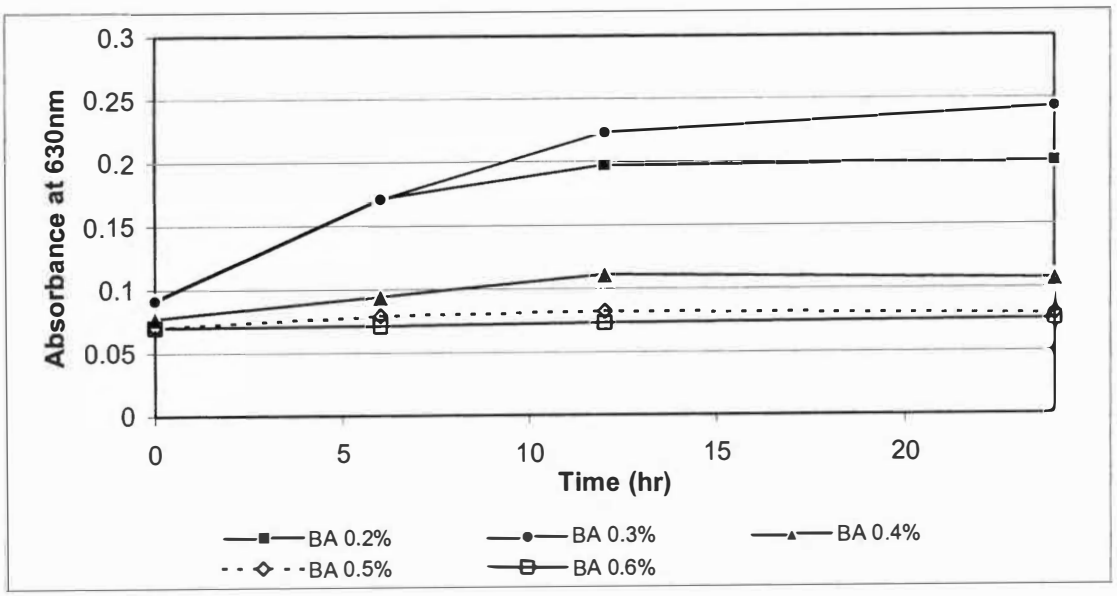
A.



B.

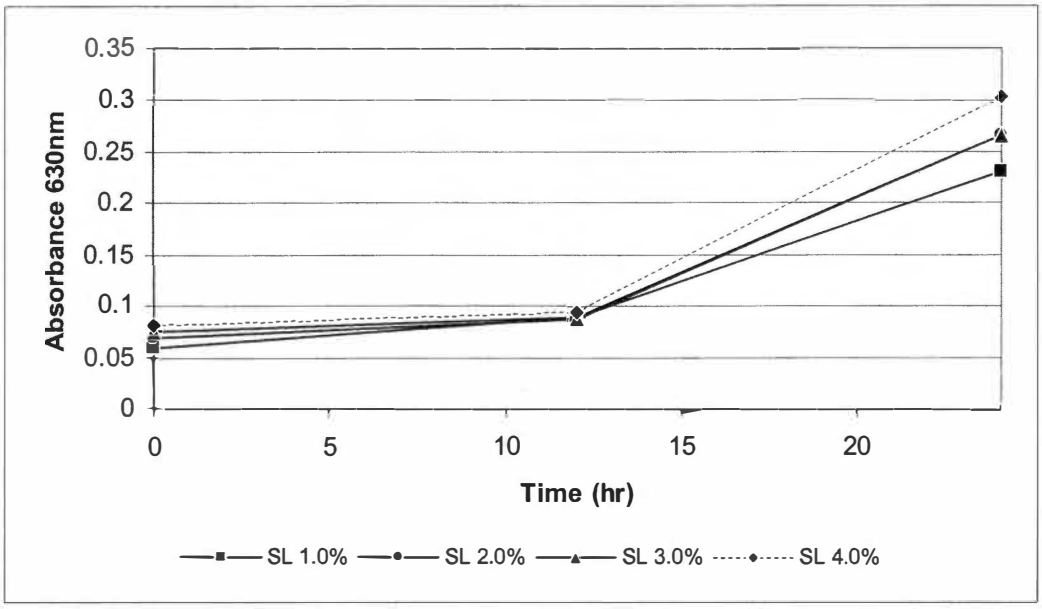


C.

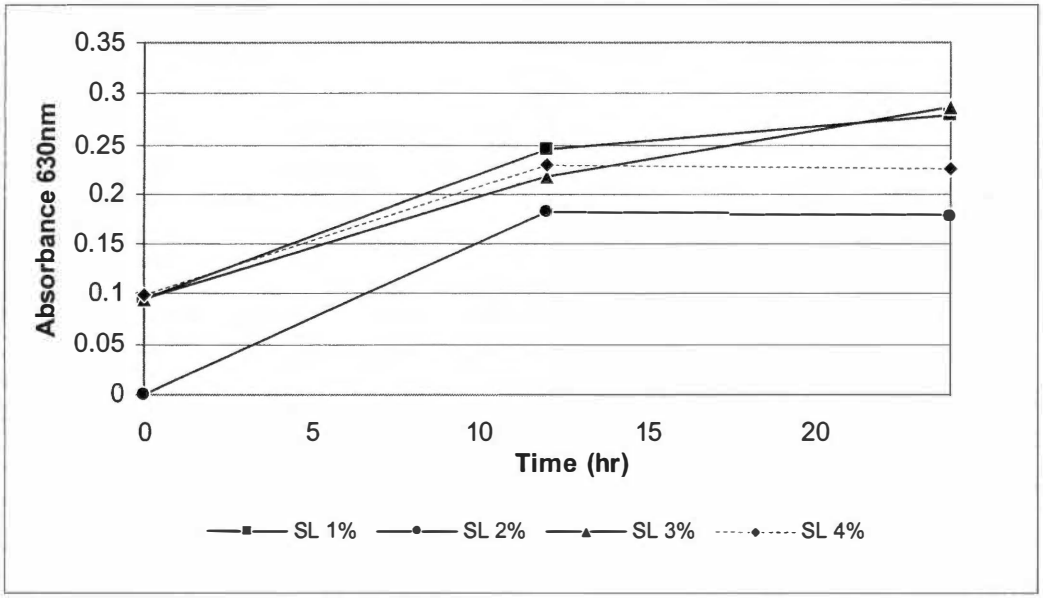

Absorbance at 630nm
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
Time (hr)
BA 0.2%
BA 0.3%
BA 0.4%
BA 0.5%
BA 0.6%

Appendix 16.0: Exposure of *L. monocytogenes* strain Scott A to sodium lactate (SL) at different concentration at pH 6.0 in 24 h.
A) First exposure B) Second exposure of cells at same initial concentration.

A.

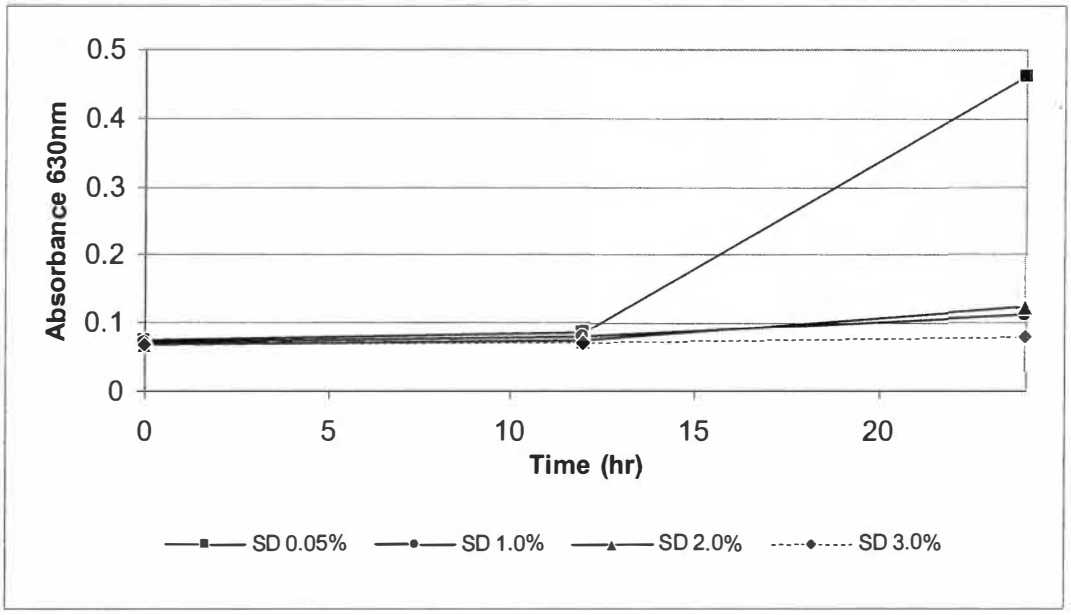


B.

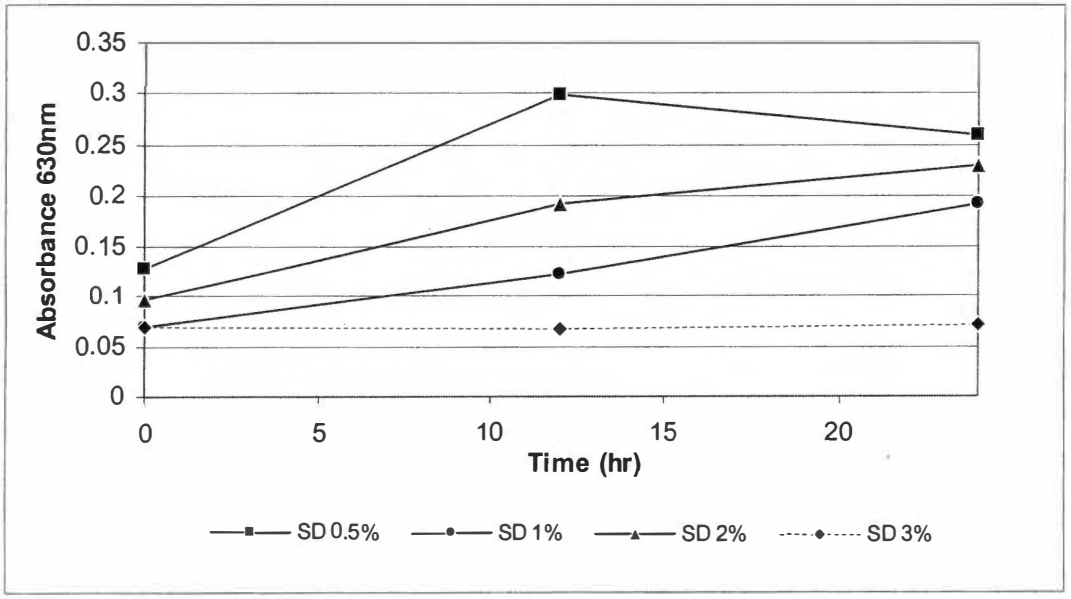


Appendix 17.0: Exposure of *L. monocytogenes* strain Scott A to sodium diacetate (SD) at different concentration at pH 6.0 in 24 h.
A) First exposure B) Second exposure of cells at same initial concentration.

A.

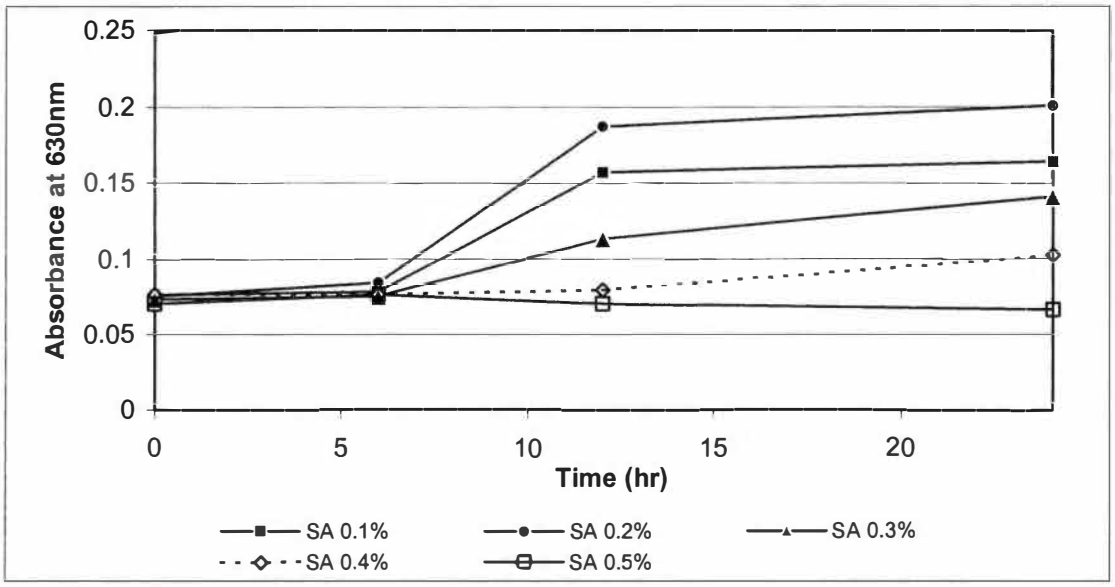


B.

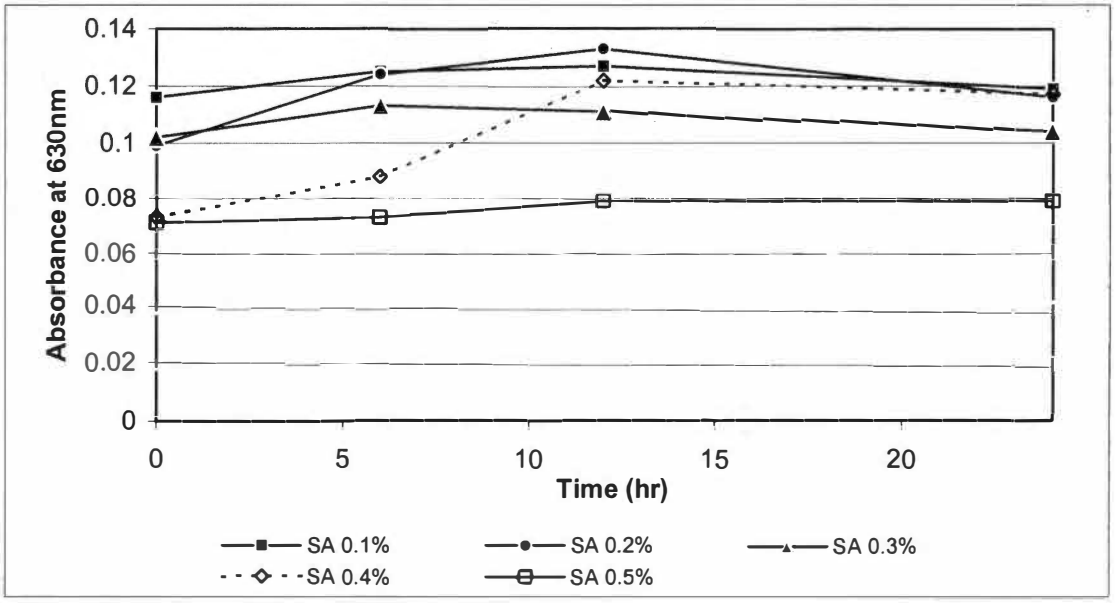


Appendix 18.0: Exposure of *S.* Typhimurium strain 2380 to sorbic acid (SA) at different concentrations at pH 6.0 in 24 h. A) Initial exposure B) Second exposure of cells at same initial concentration. C) Third exposure at one step higher concentration.

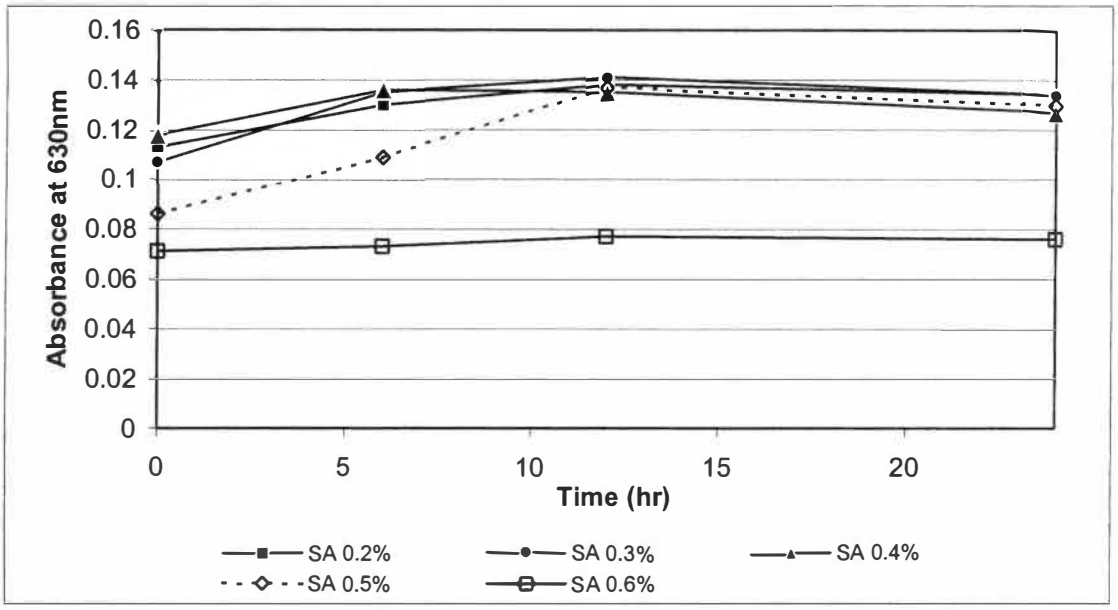
A.



B.

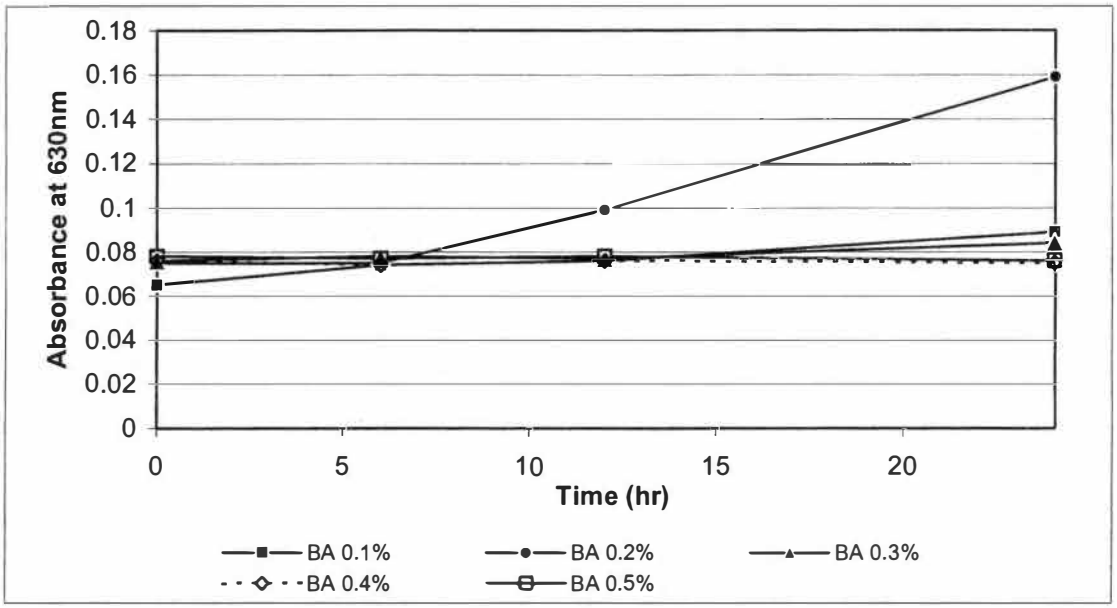


C.

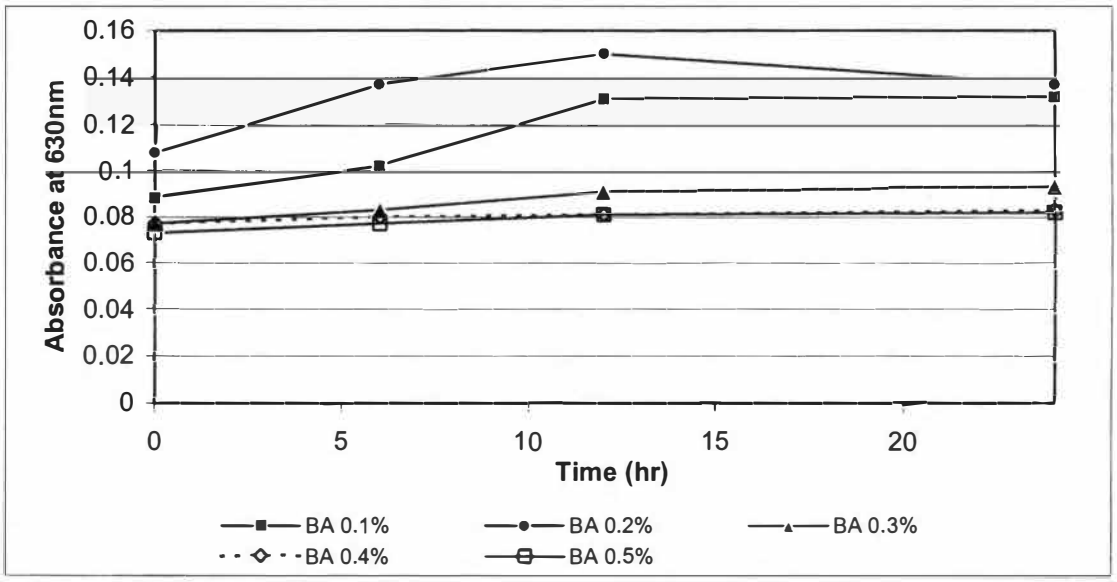

Absorbance at 630nm
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
5
10
15
20
Time (hr)
SA 0.2%
SA 0.3%
SA 0.4%
SA 0.5%
SA 0.6%

Appendix 19.0: Exposure of *S.* Typhimurium strain 2380 to benzoic acid (BA) at different concentrations at pH 6.0 in 24 h. A) Initial exposure B) Second exposure of cells at same initial concentration. C) Third exposure at one step higher concentration.

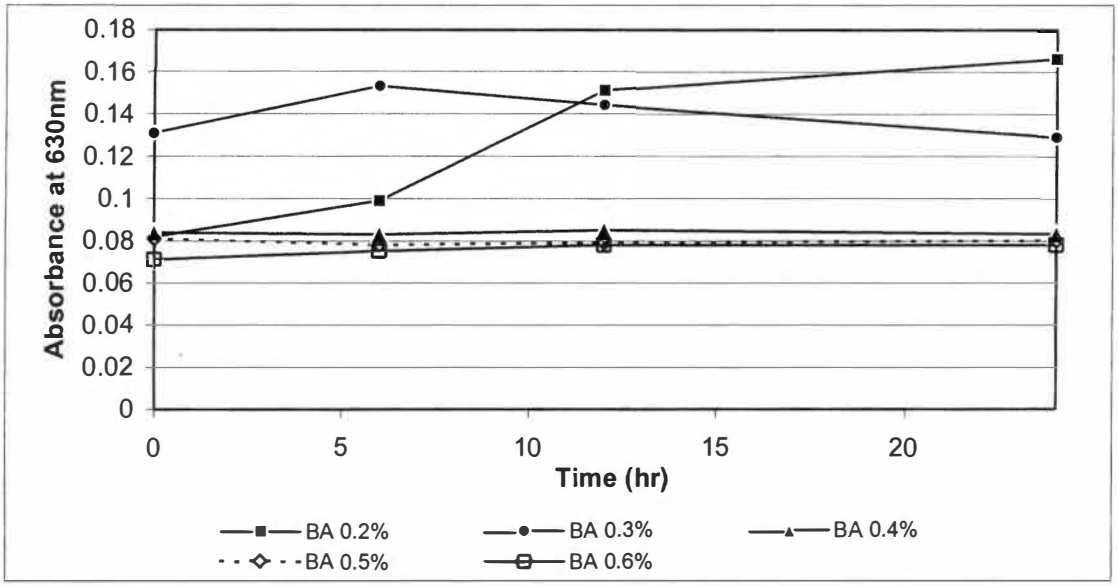
A.



B.

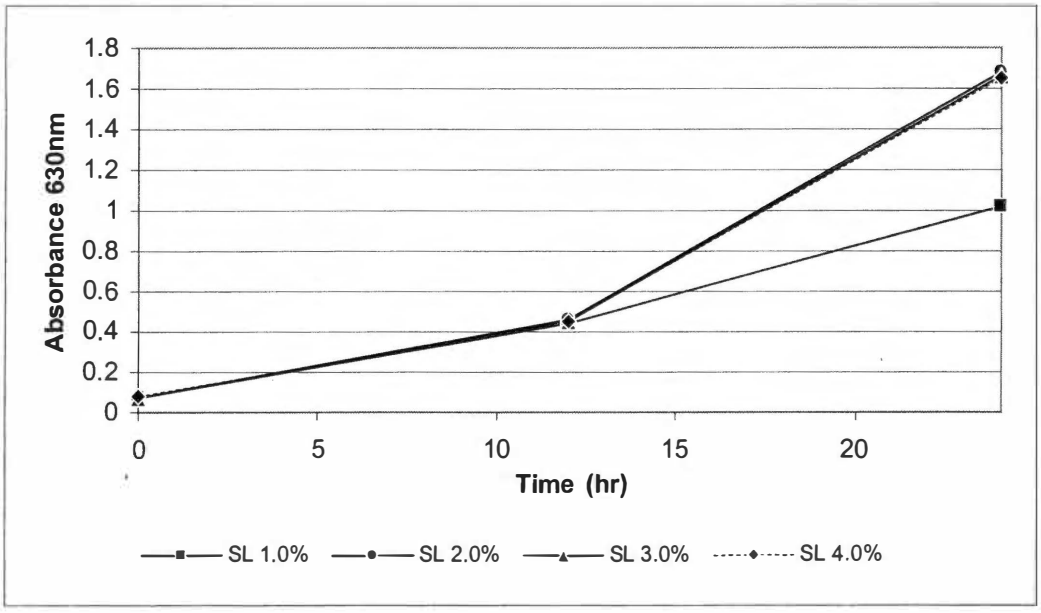


C.

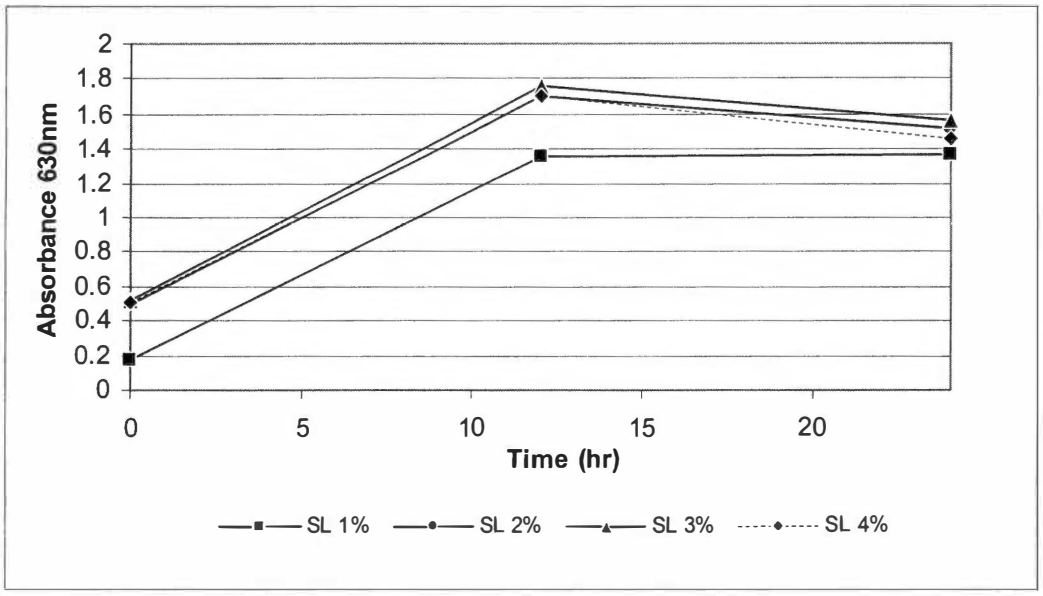

Absorbance at 630nm
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
5
10
15
20
Time (hr)
BA 0.2%
BA 0.3%
BA 0.4%
BA 0.5%
BA 0.6%

Appendix 20.0: Exposure of *S.* Typhimurium strain 2380 to sodium lactate (SL) at different concentration at pH 6.0 in 24 h.
A) First exposure B) Second exposure of cells at same initial concentration.

A.

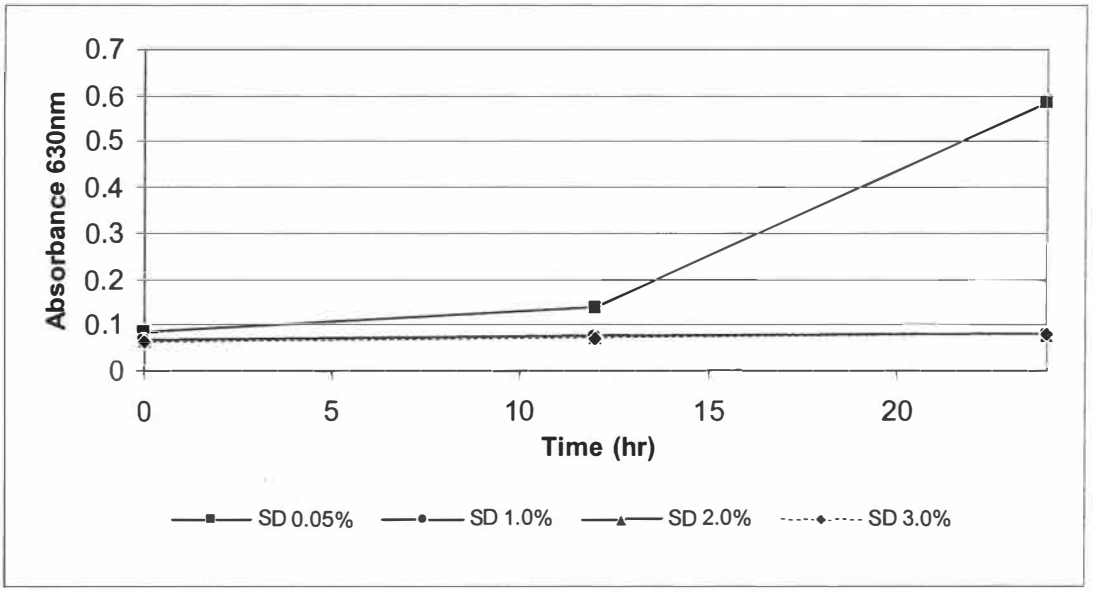


B.

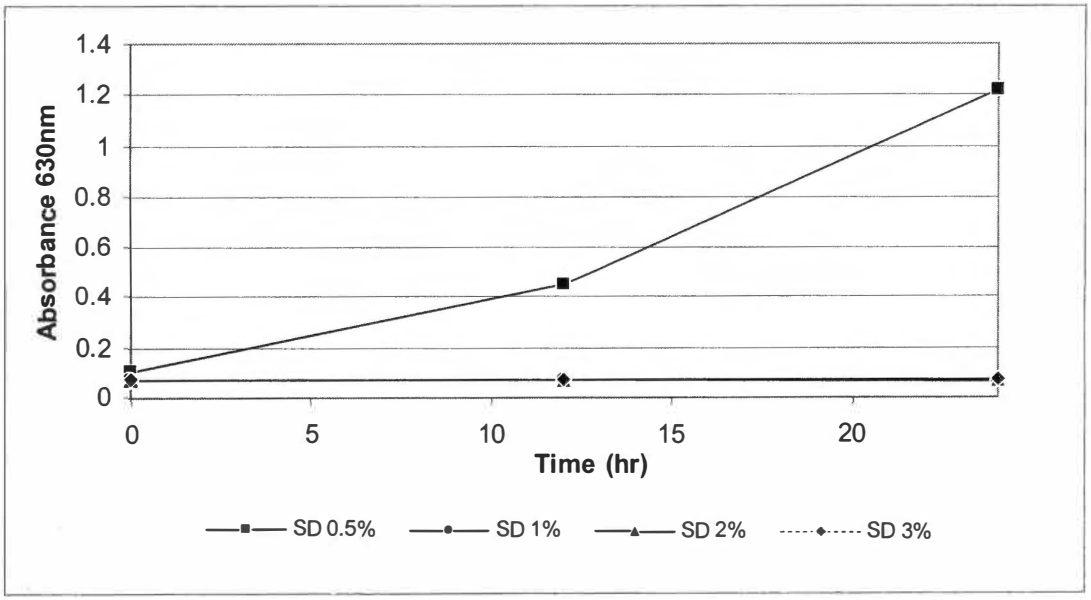


Appendix 21.0: Exposure of *S.* Typhimurium strain 2380 to sodium diacetate (SD) at different concentration at pH 6.0 in 24 h.
A) First exposure B) Second exposure of cells at same initial concentration.

A.

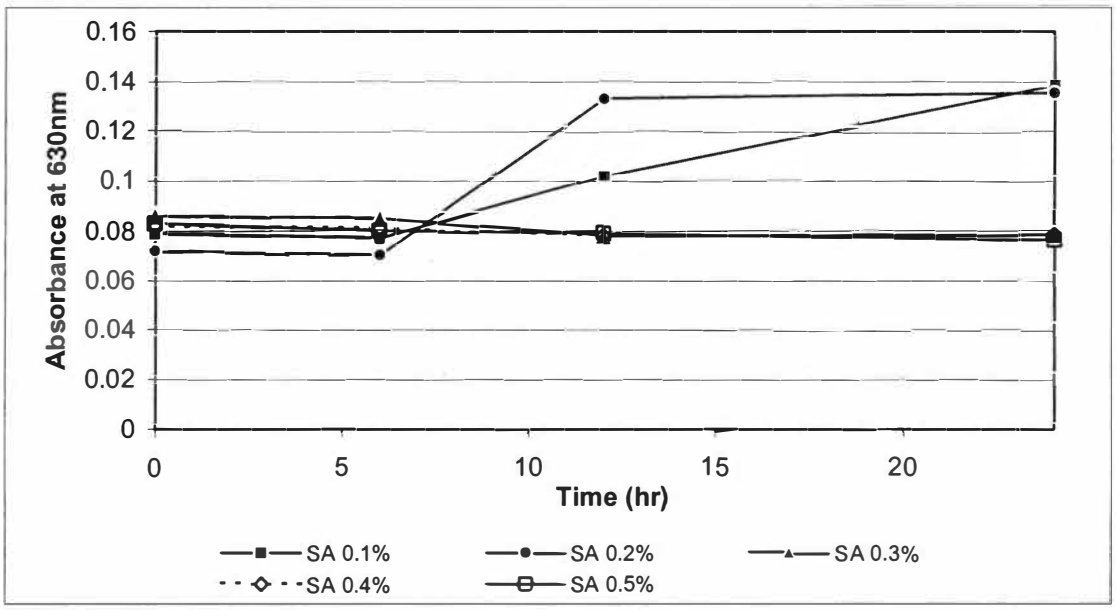


B.

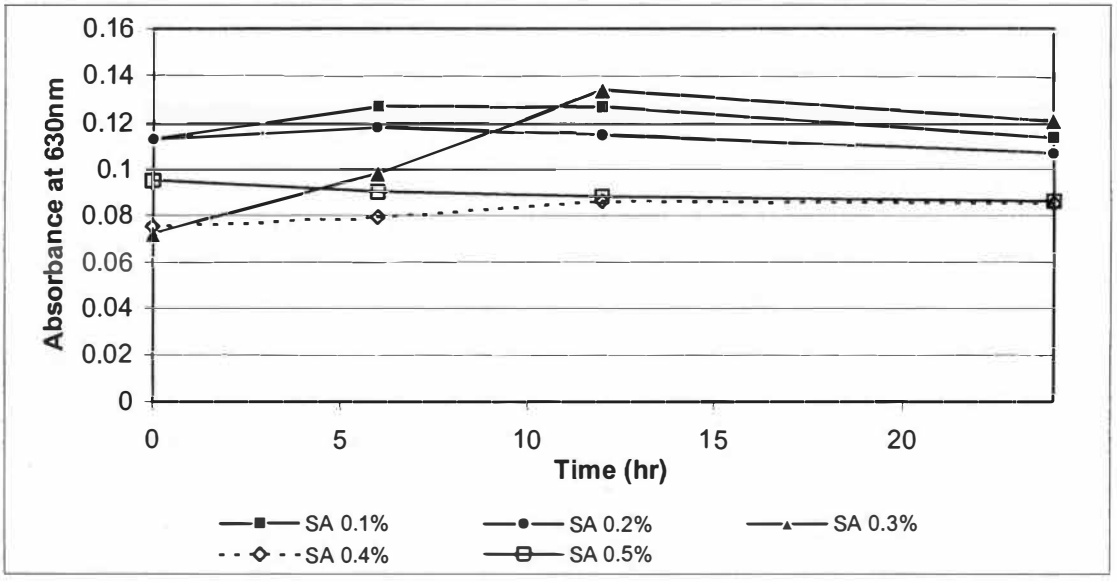


Appendix 22.0: Exposure of *S.* Typhimurium strain 2486 to sorbic acid (SA) at different concentrations at pH 6.0 in 24 h. A) Initial exposure B) Second exposure of cells at same initial concentration. C) Third exposure at one step higher concentration.

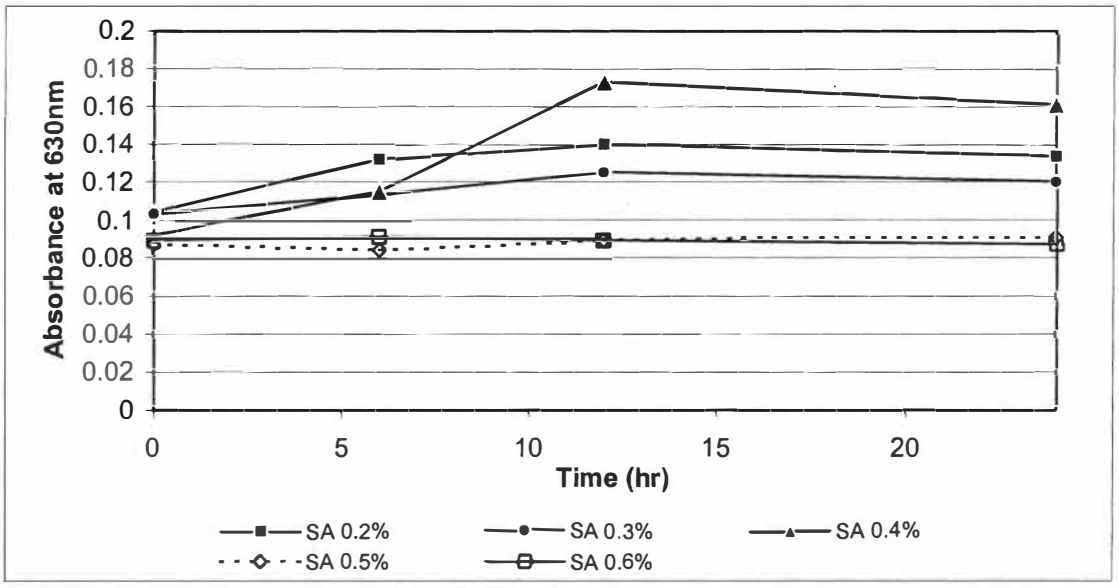
A.



B.

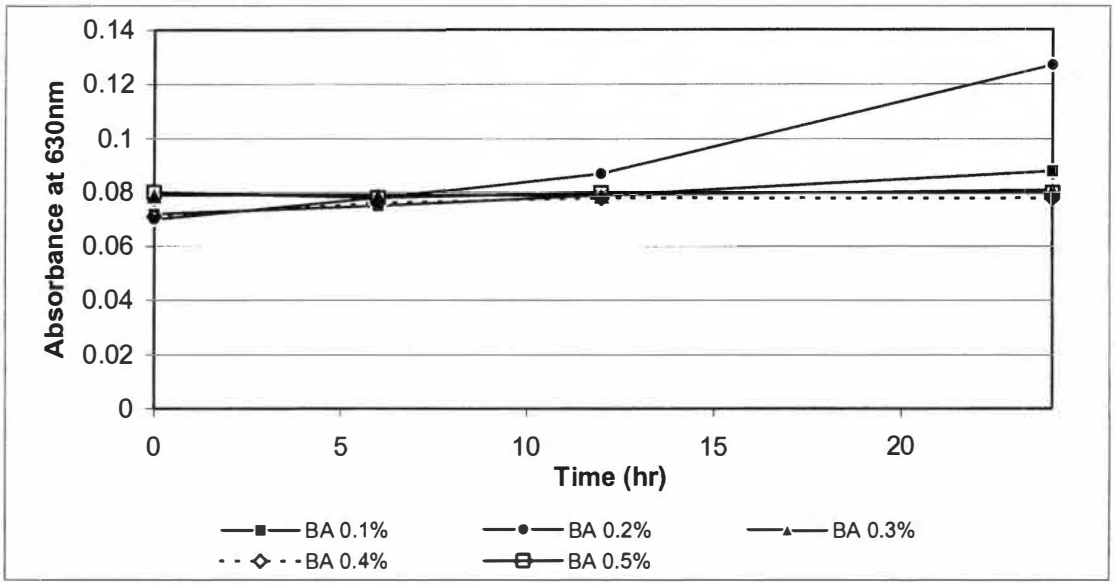


C.

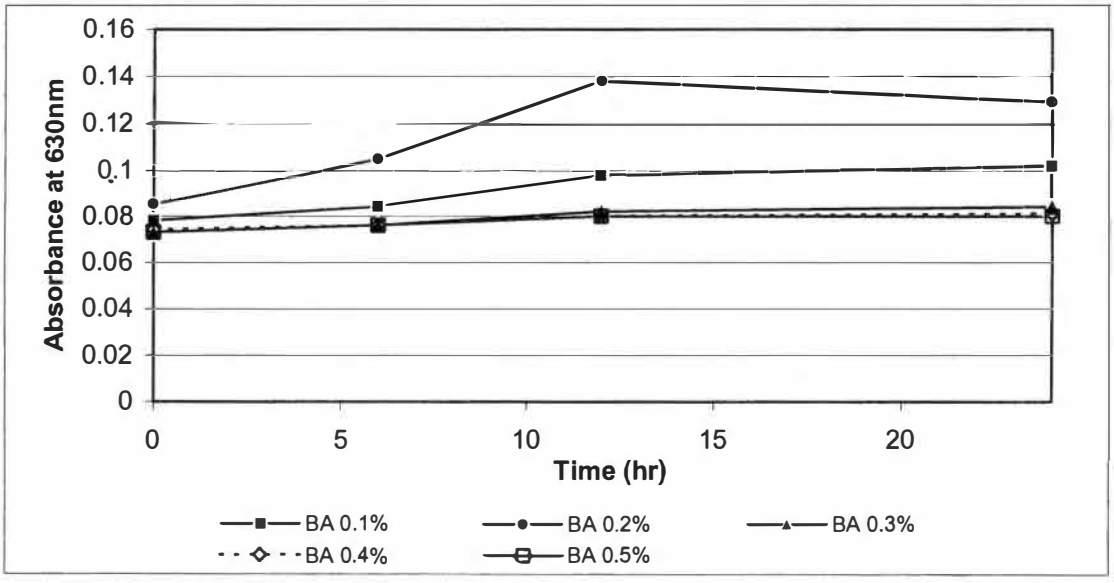

Absorbance at 630nm
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
5
10
15
20
Time (hr)
SA 0.2%
SA 0.3%
SA 0.4%
SA 0.5%
SA 0.6%

Appendix 23.0: Exposure of *S.* Typhimurium strain 2486 to benzoic acid (BA) at different concentration at pH 6.0 in 24 h. A) Initial exposure B) Second exposure of cells at same initial concentration. C) Third exposure at one step higher concentration.

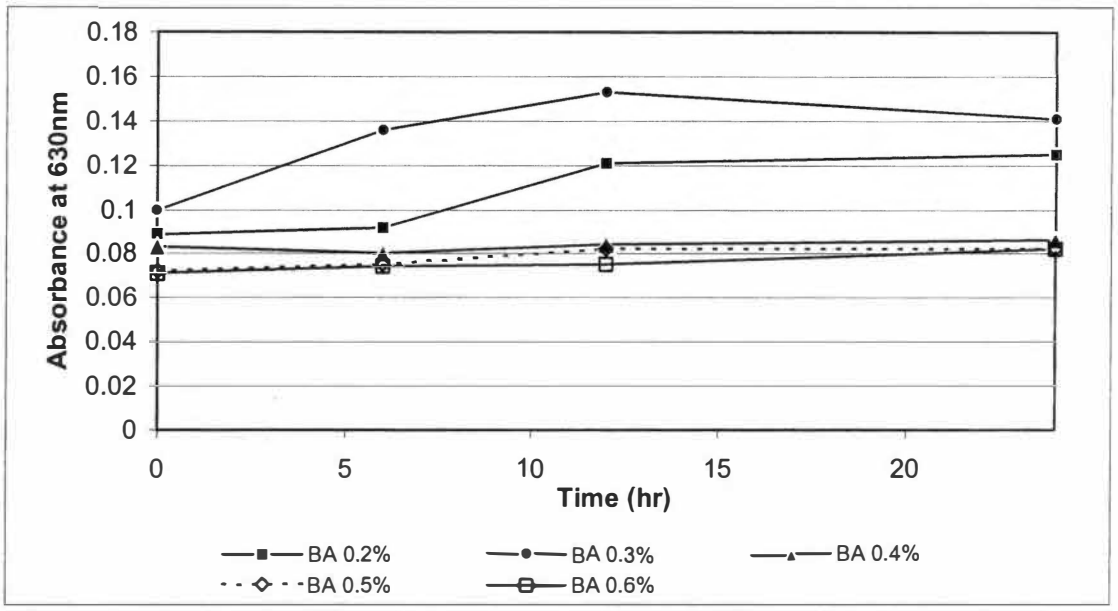
A.



B.

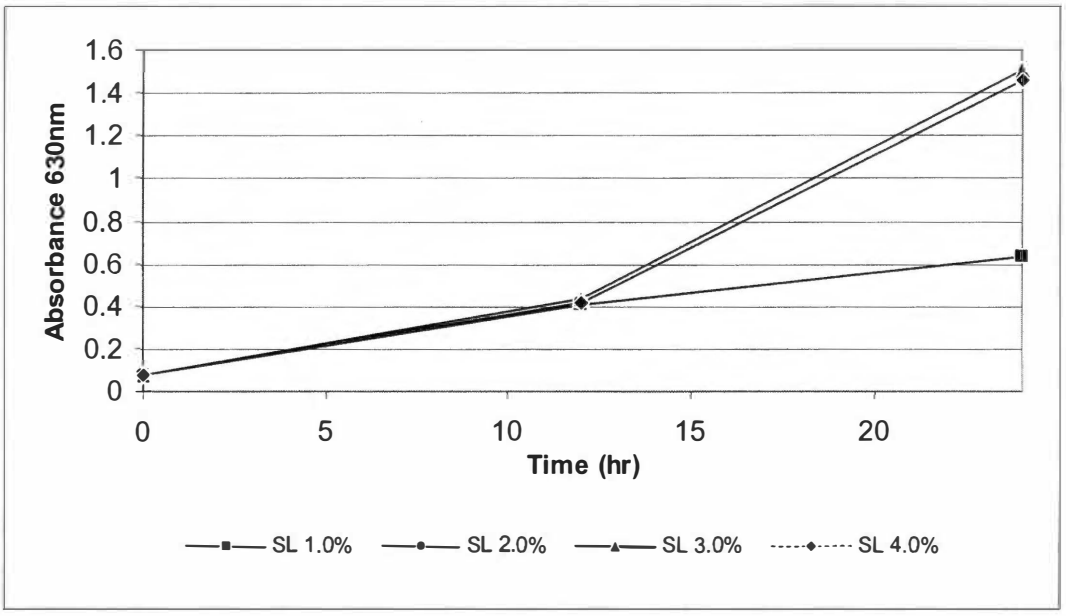


C.

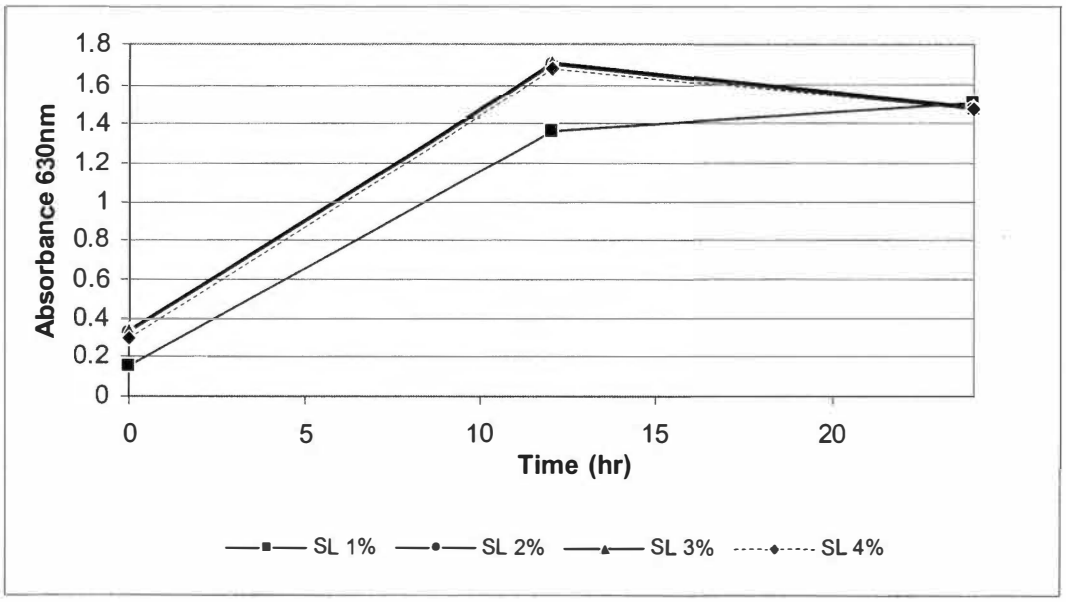

Absorbance at 630nm
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
5
10
15
20
Time (hr)
BA 0.2%
BA 0.3%
BA 0.4%
BA 0.5%
BA 0.6%

Appendix 24.0: Exposure of *S.* Typhimurium strain 2486 to sodium lactate (SL) at different concentration at pH 6.0 in 24 h.
A) First exposure B) Second exposure of cells at same initial concentration.

A.

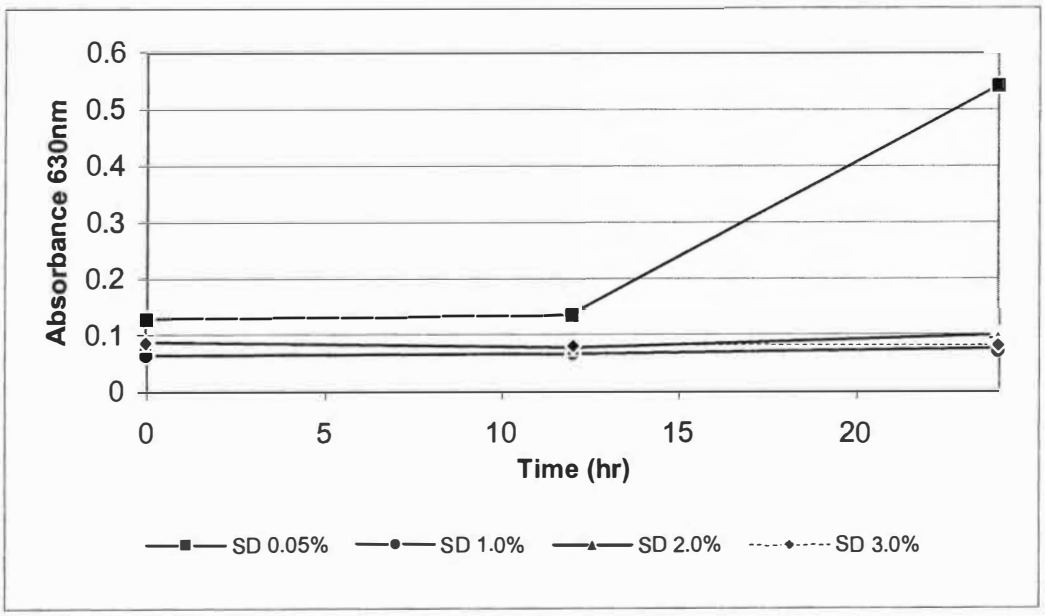


B.

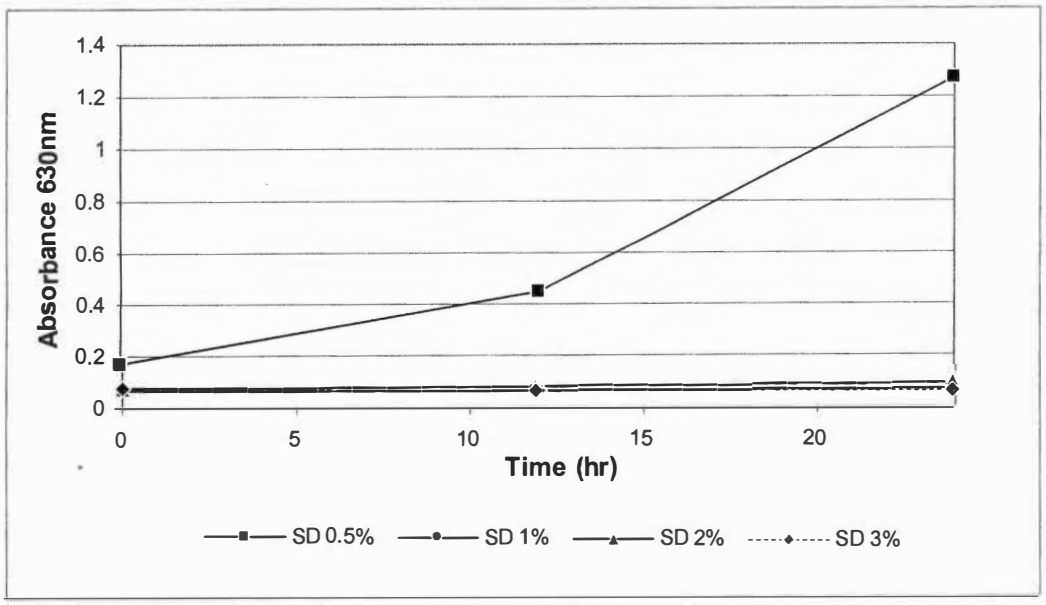


Appendix 25.0: Exposure of *S*. Typhimurium strain 2486 to sodium diacetate (SD) at different concentration at pH 6.0 in 24 h.
A) First exposure B) Second exposure of cells at same initial concentration.

A.

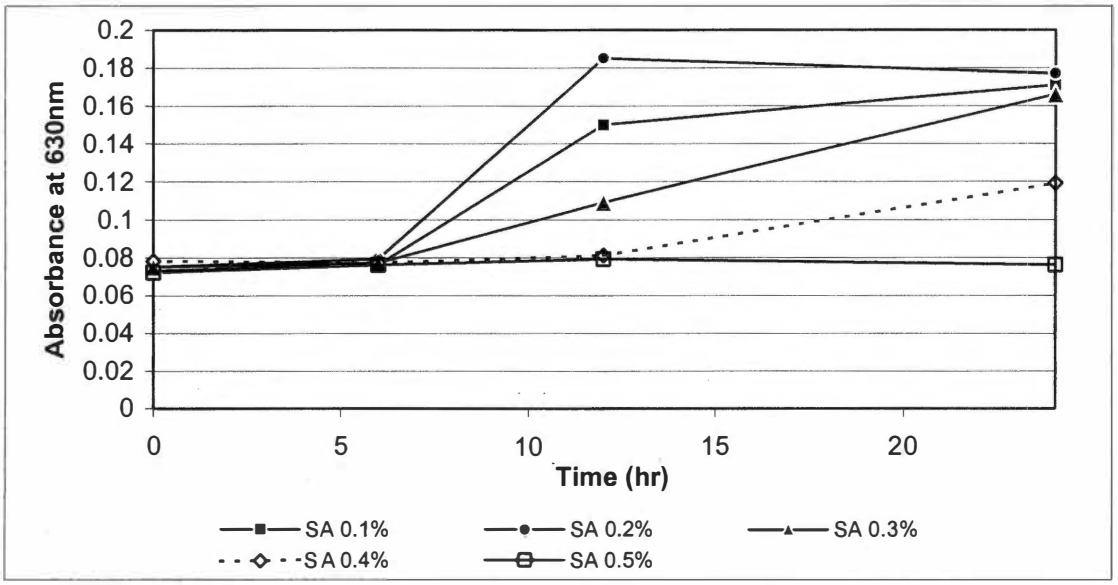


B.

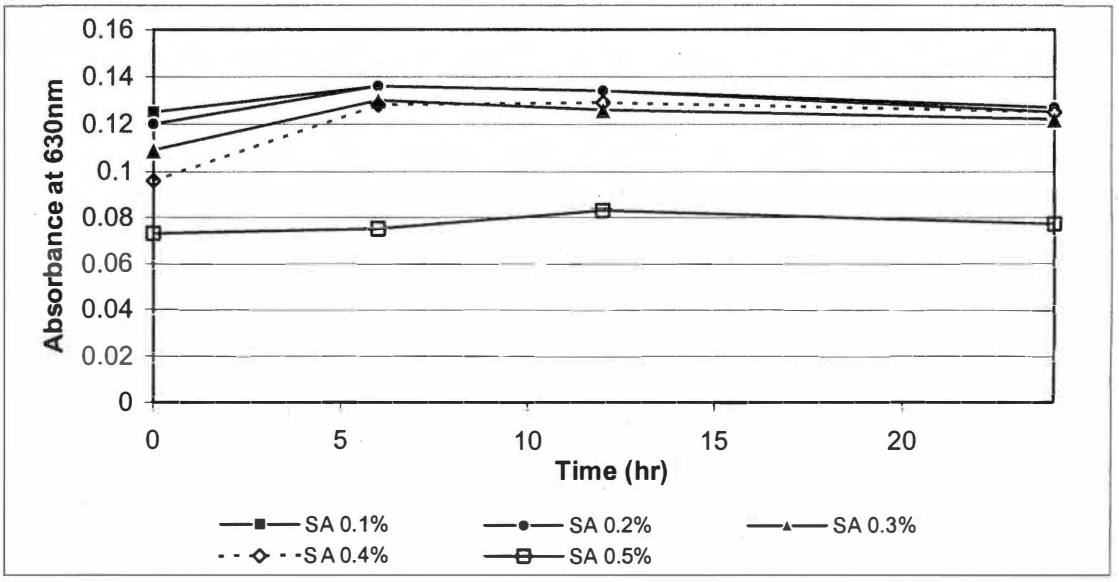


Appendix 26.0: Exposure of *S.* Typhimurium strain 2576 to sorbic acid (SA) at different concentration at pH 6.0 in 24 h. A) Initial exposure B) Second exposure of cells at same initial concentration. C) Third exposure at one step higher concentration.

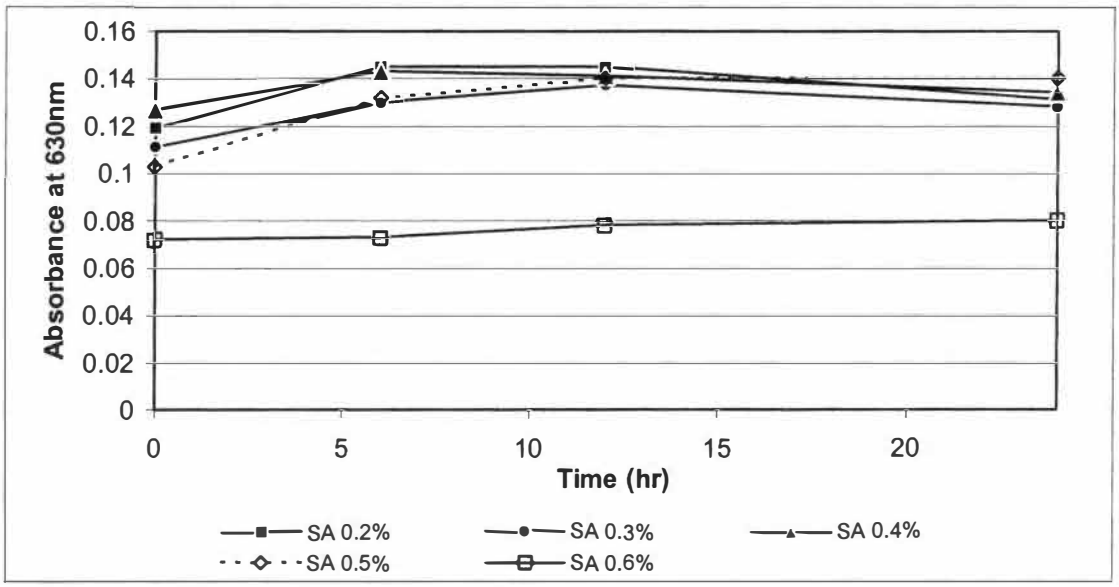
A.



B.

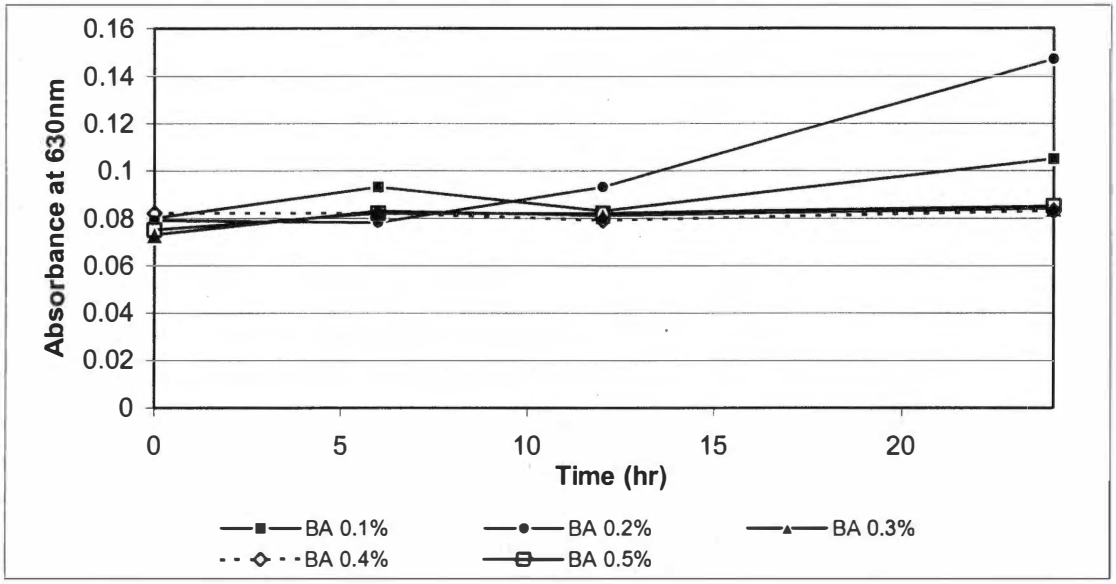


C.

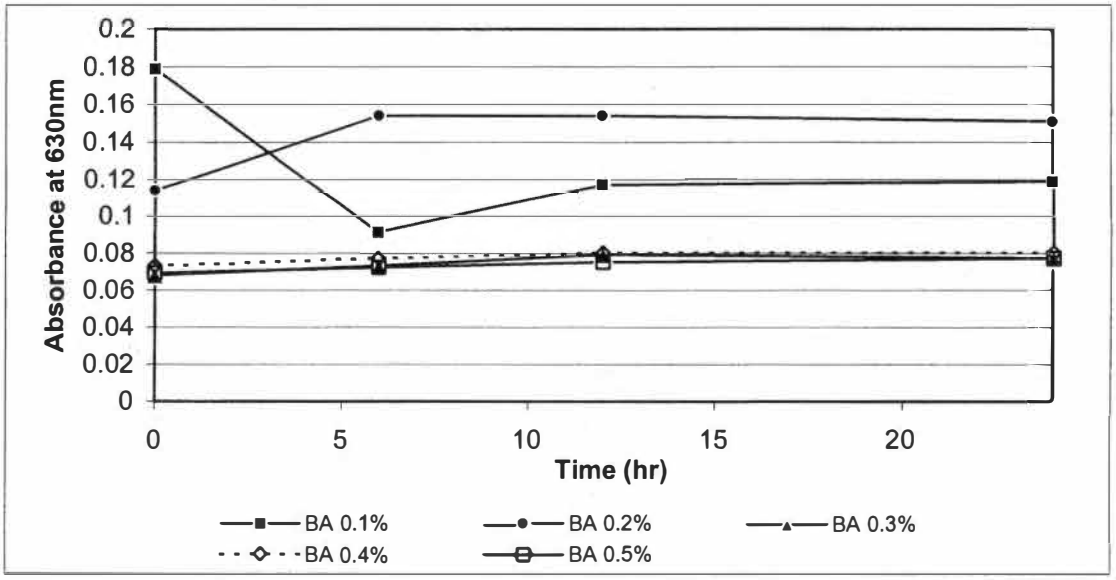

0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Absorbance at 630nm
0
5
10
15
20
Time (hr)
SA 0.2%
SA 0.3%
SA 0.4%
SA 0.5%
SA 0.6%

Appendix 27.0: Exposure of *S.* Typhimurium strain 2576 to benzoic acid (BA) at different concentrations at pH 6.0 in 24 h. A) Initial exposure B) Second exposure of cells at same initial concentration. C) Third exposure at one step higher concentration.

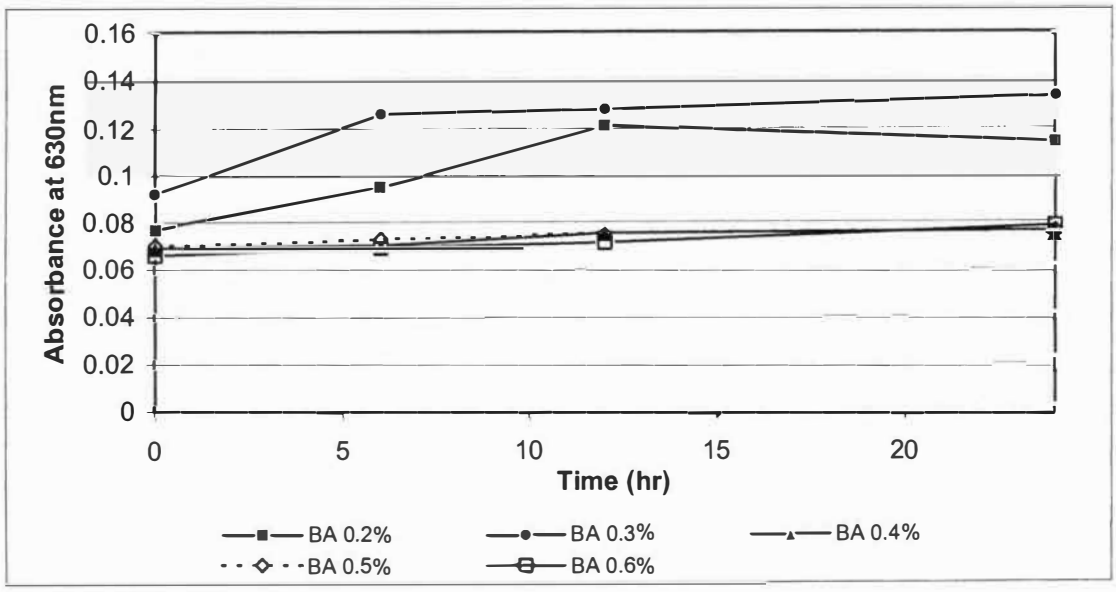
A.



B.

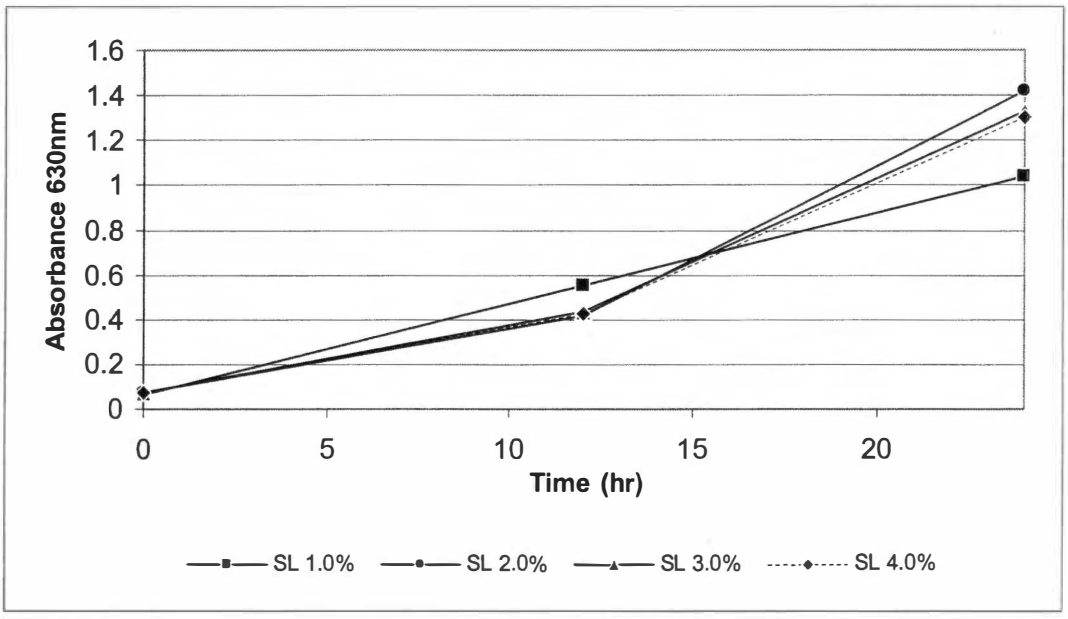


C.

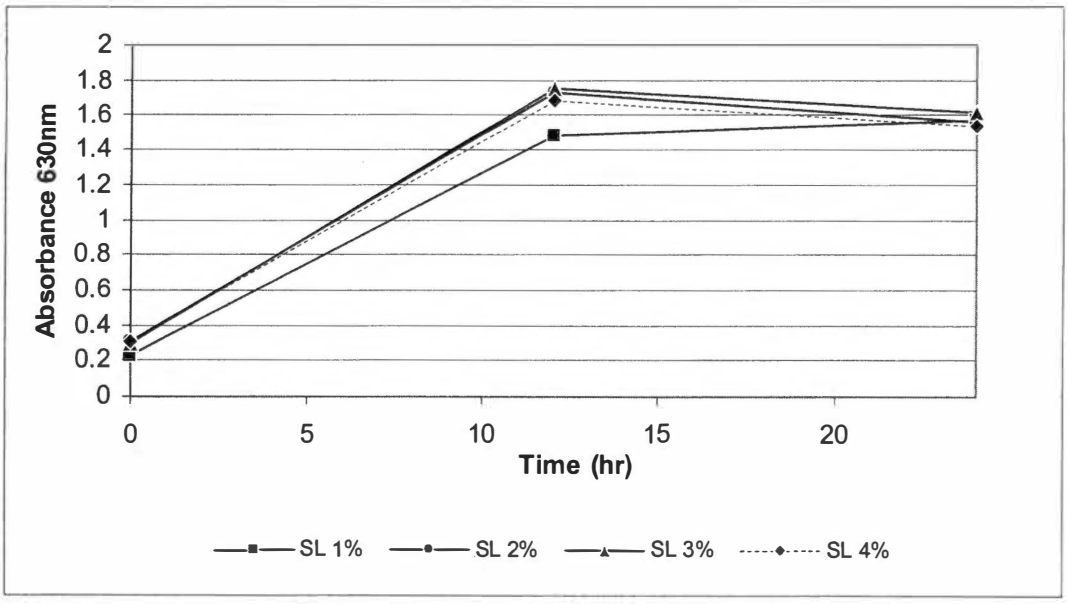

0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Absorbance at 630nm
0
5
10
15
20
Time (hr)
BA 0.2%
BA 0.3%
BA 0.4%
BA 0.5%
BA 0.6%

Appendix 28.0: Exposure of S. Typhimurium strain 2576 to sodium lactate (SL) at different concentration at pH 6.0 in 24 h.
A) First exposure B) Second exposure of cells at same initial concentration.

A.

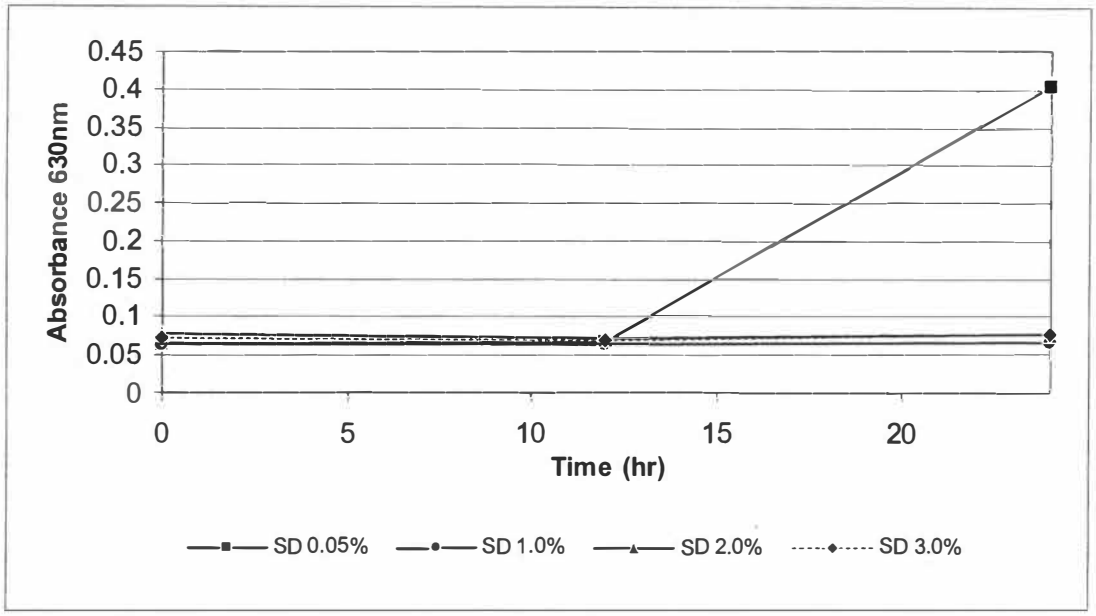


B.

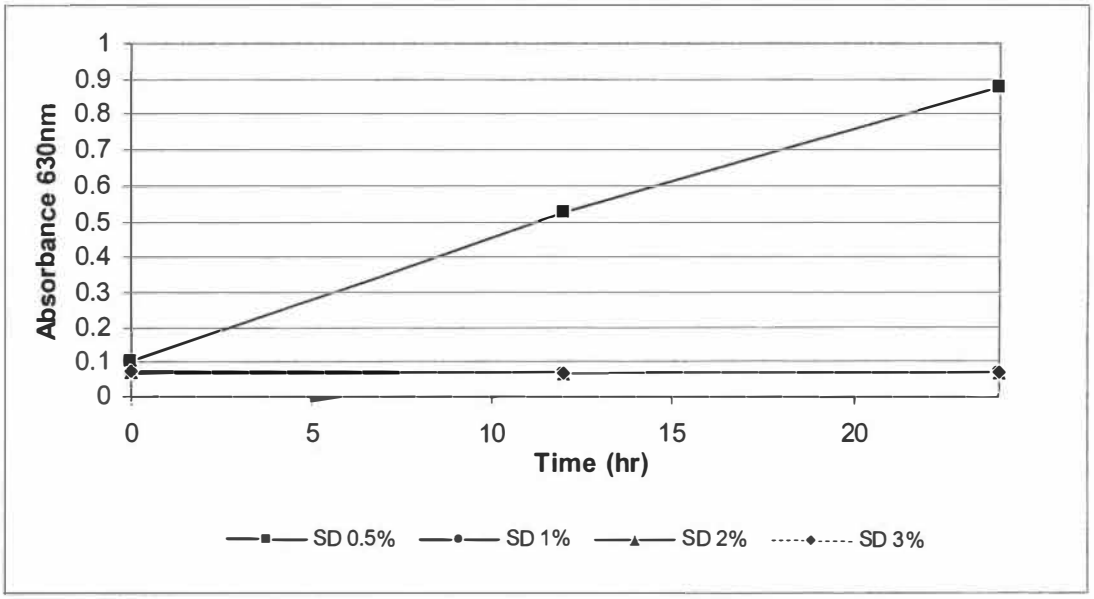


Appendix 29.0: Exposure of *S.* Typhimurium strain 2576 to sodium diacetate (SD) at different concentration at pH 6.0 in 24 h.
A) First exposure B) Second exposure of cells at same initial concentration.

A.

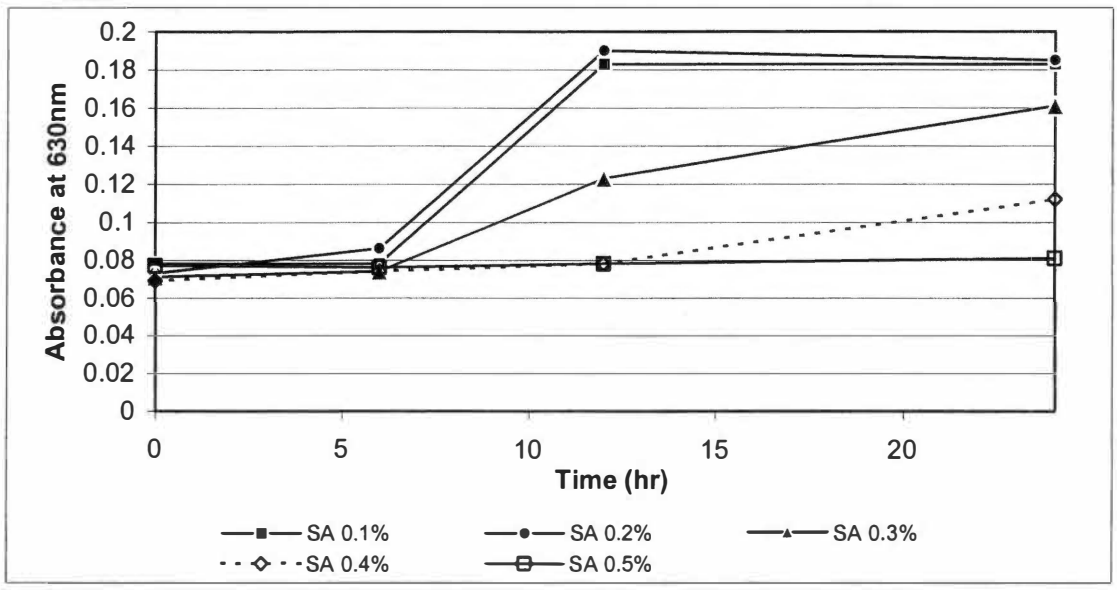


B.

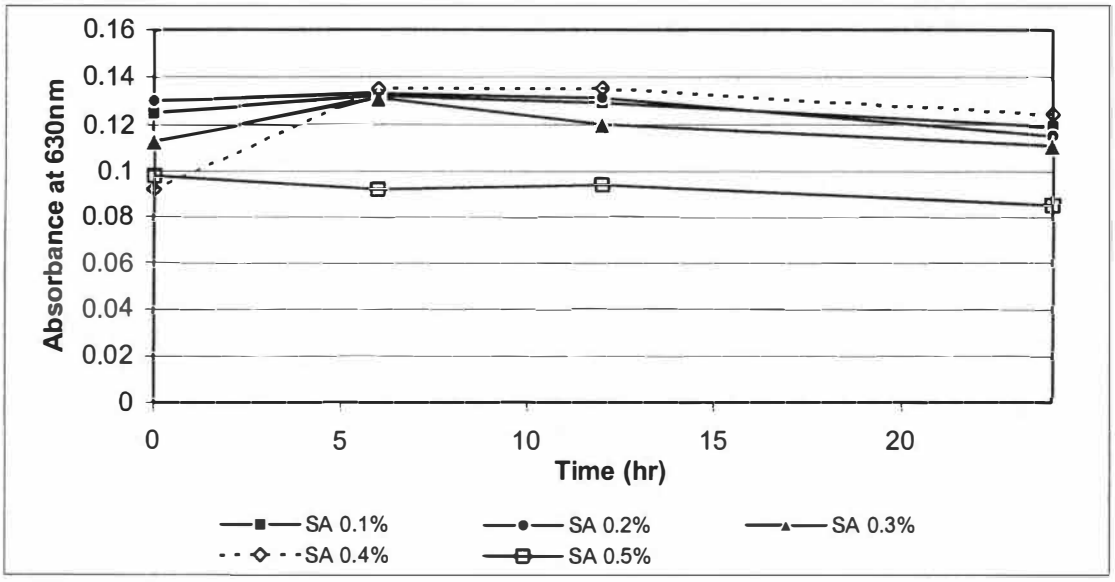


Appendix 30.0: Exposure of *S.* Typhimurium strain 2582 to sorbic acid (SA) at different concentrations at pH 6.0 in 24 h. A) Initial exposure B) Second exposure of cells at same initial concentration. C) Third exposure at one step higher concentration.

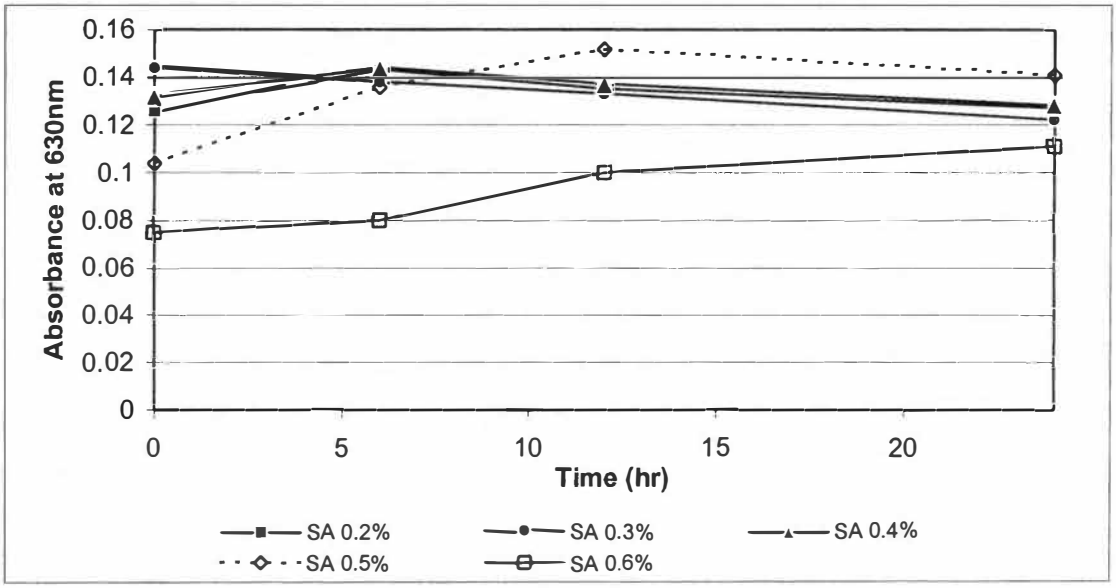
A.



B.

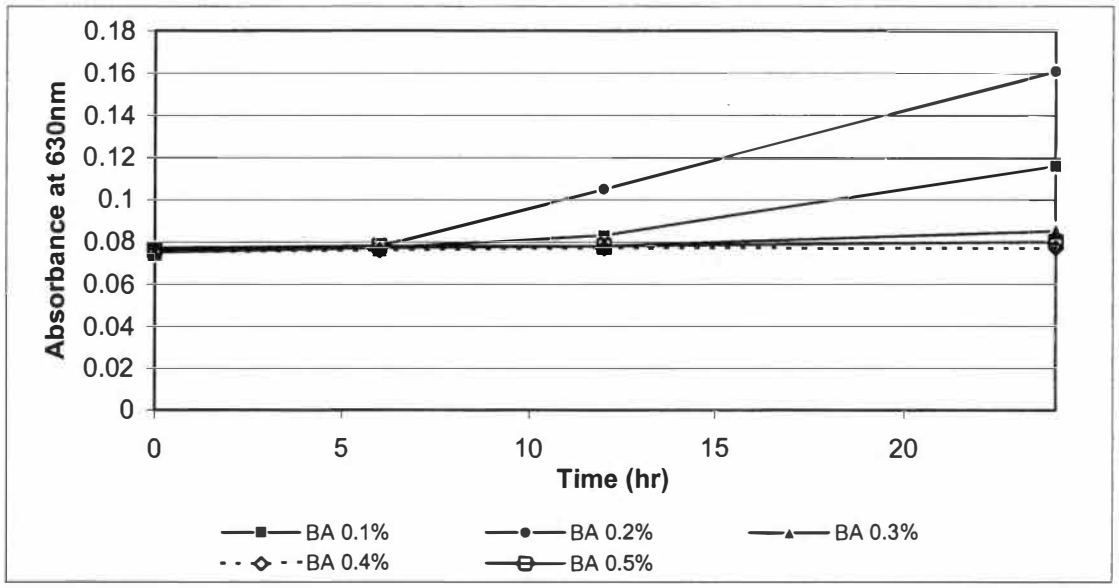


C.

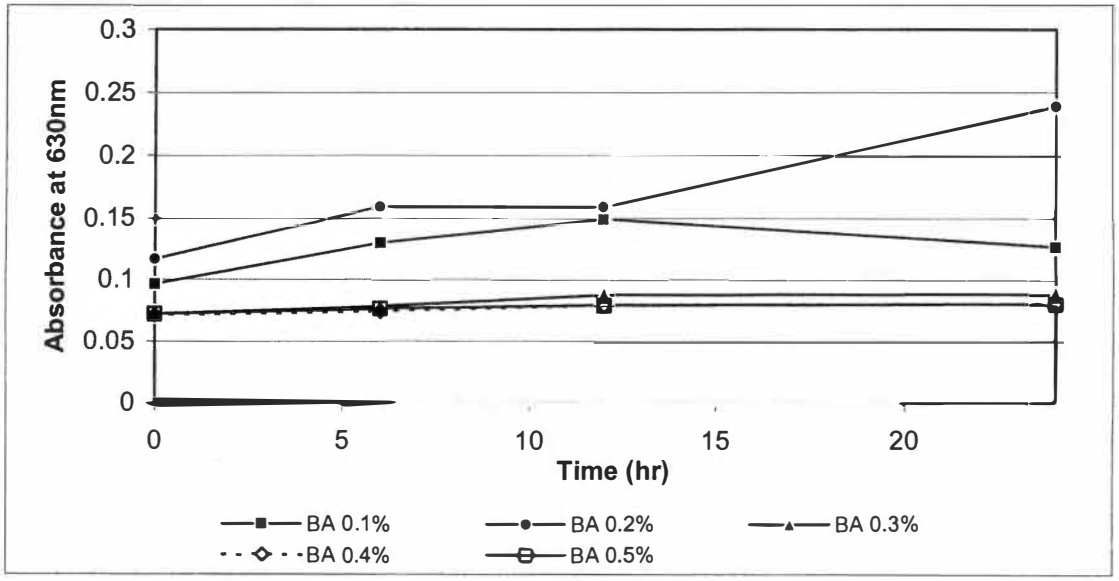

0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Absorbance at 630nm
0
5
10
15
20
Time (hr)
SA 0.2%
SA 0.3%
SA 0.4%
SA 0.5%
SA 0.6%

Appendix 31.0: Exposure of *S.* Typhimurium strain 2582 to benzoic acid (BA) at different concentrations at pH 6.0 in 24 h. A) Initial exposure B) Second exposure of cells at same initial concentration. C) Third exposure at one step higher concentration.

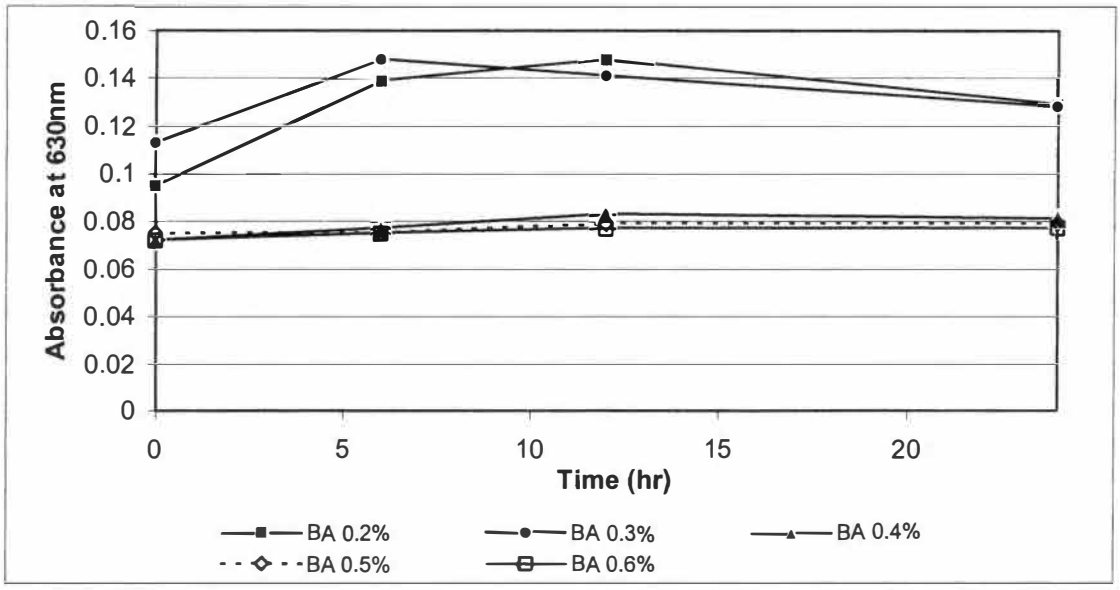
A.



B.

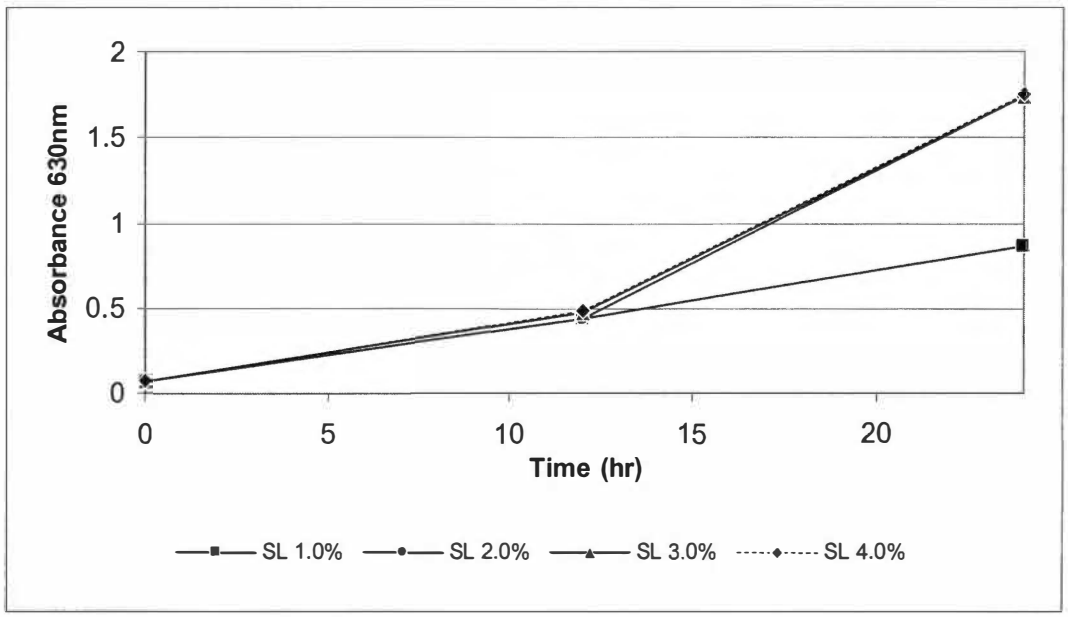


C.

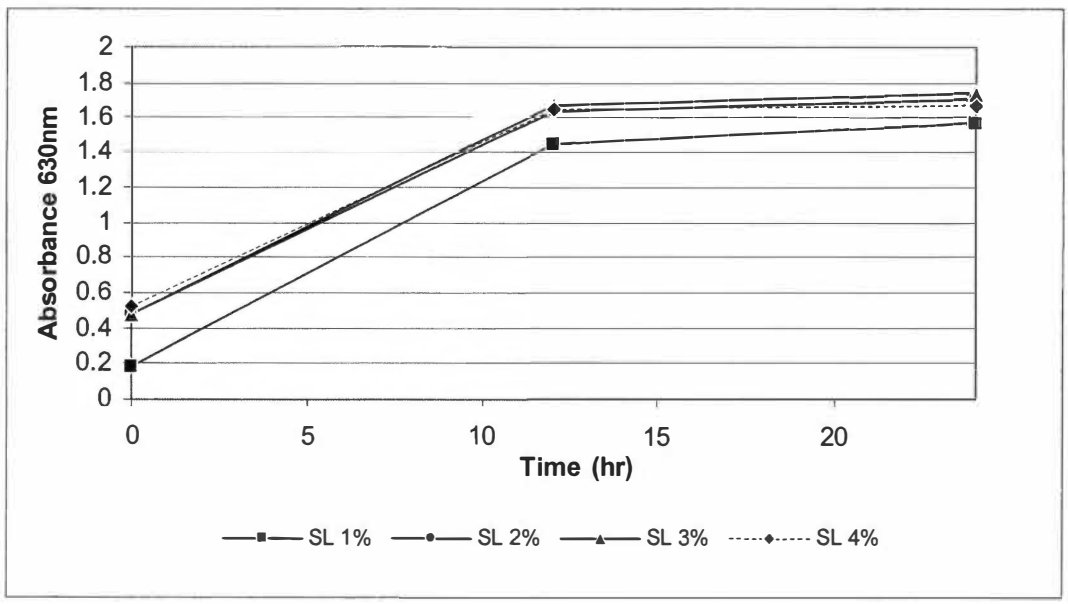

0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Absorbance at 630nm
0
5
10
15
20
Time (hr)
BA 0.2%
BA 0.3%
BA 0.4%
BA 0.5%
BA 0.6%

Appendix 32.0: Exposure of *S.* Typhimurium strain 2582 to sodium lactate (SL) at different concentration at pH 6.0 in 24 h.
A) First exposure B) Second exposure of cells at same initial concentration.

A.

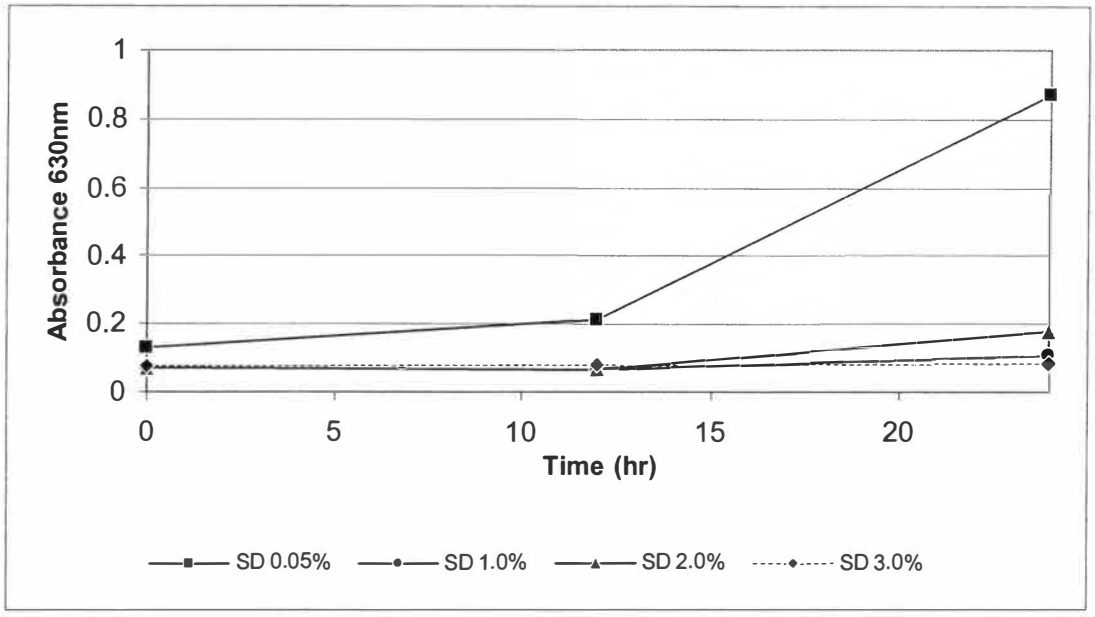


B.

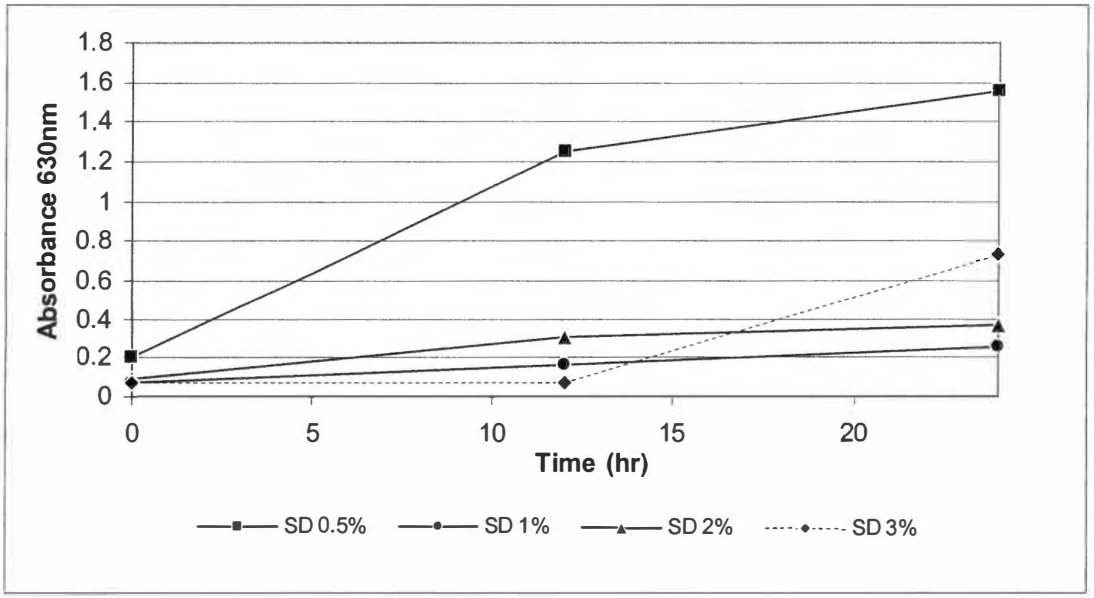


Appendix 33.0: Exposure of *S*. Typhimurium strain 2582 to sodium diacetate (SD) at different concentration at pH 6.0 in 24 h.
A) First exposure B) Second exposure of cells at same initial concentration.

A.



B.



PART THREE: GROWTH EVALUATION OF *LISTERIA MONOCYTOGENES* AND *SALMONELLA* TYPHIMURIUM ADAPTED TO INCREASING CONCENTRATIONS OF FOOD ANTIMICROBIALS AND CULTURED IN DIFFERENT MEDIUM ENVIRONMENTS

Abstract

The growth susceptibility of two strains of *Listeria monocytogenes* (101, 108) and *Salmonella* Typhimurium (2380, 2486) adapted to increasing concentrations of potassium sorbate (PS), sodium benzoate (SB), sodium lactate (SL) and sodium diacetate (SD) at pH 6.0 was studied. Cells were not subjected to any pre-stress condition. Two different media were used to determine if the presence of glucose affected resistance to antimicrobials. An agar dilution assay was performed to adapt cells to increasing antimicrobial concentrations. Concentrations for PS and BS ranged from 0.1 to 0.4%, for SL 1.0 to 4.0% and for SD 0.5 to 0.2%. A growth curve was made to compare growth of adapted vs. non-adapted (parent) strains at same high concentrations. Adapted strains grown in TSB with and without glucose resulted in higher log CFU/ml compared to non-adapted strains when exposed to previous and higher antimicrobial concentrations. Adapted and non-adapted strains were more susceptibility when exposed to higher concentrations and grown in TSB with no glucose indicating that presence of glucose affected tolerance to antimicrobials. Adapted and non-adapted were more susceptible to higher concentrations of benzoate (0.5%) and diacetate (1.0%) when grown in TSB with no glucose. Results indicated that adapted *L. monocytogenes* and *S.* Typhimurium strains to regulatory-approved food antimicrobials are more resistant than non-adapted strains when exposed to same or higher antimicrobial concentrations.

I. Introduction

Food antimicrobials are chemical compounds used to extend the lag phase or kill microorganisms. They are added directly to food or, as sprays or dips for inhibition or inactivation of microorganisms (Davidson and Harrison, 2003). Food antimicrobials may be classified into two groups: traditional or "regulatory approved" and naturally occurring (Davidson and Harrison 2002). Some traditional antimicrobials include acetates, sorbates, benzoates, lactates, propionates, and nitrites and nitrates. For many years, food antimicrobials were used primarily to prolong shelf life and preserve quality of foods through inhibition of spoilage microorganisms. Recently, food processors have been increasingly using antimicrobials to inhibit or inactivate pathogenic microorganisms in foods. Historically, the only food antimicrobial used exclusively to inhibit a pathogen in a food has been nitrite, which is used to inhibit *Clostridium botulinum* in cured meats (Davidson and Harrison, 2003). More recently antimicrobials such as lactates and diacetates have been added to processed meats or spray sanitizers on beef carcasses to inactivate pathogens such as *L. monocytogenes* (FDA, 2000).

Recent studies report that the direct use of certain food antimicrobials may impose a selective pressure and contribute to the emergence of resistant microorganism in food environments (Russell, 2000). In the food-processing environment, bacteria are exposed to multiple stresses (e.g., heat, antimicrobials compounds), which are mainly used to preserve quality, increase shelf life and improve safety of food products. Sublethal preservation stresses may result in cells that are less susceptible to subsequent stresses (Hill and Gahan, 2000; Ravishankar and Juneja, 2003). Under such conditions, bacteria

present in the plant environment survive and may be able to adapt to even harsher treatments (Ravishankar and Juneja, 2003).

Bacterial resistance to any type of antimicrobial compound may be mediated by multiple mechanisms and/or resistance determinants in the bacteria cell. Some cells will grow and survive after antimicrobial exposure because they may possess a degree of natural resistance, or may acquire it later through mutation or genetic exchange (Bower and Daeschel, 1999).

There is little data or evidence that foodborne bacteria develop resistance to most traditional food antimicrobials. Still, it is important to consider that if antimicrobials are to be used for exclusive control of foodborne pathogens, the potential for development of adapted strains should be evaluated (Davidson and Harrison, 2003). Resistant pathogens surviving traditional regulatory-approved food antimicrobials and sanitizers represent a threat to the food industry and consumers. It is crucial to investigate the potential for resistance development and monitor the proper and adequate use of antimicrobial compounds in order to preserve the safety of the food supply.

Therefore, the purpose of this study was to investigate whether foodborne pathogens could become resistant to traditional, regulatory-approved food antimicrobials such as potassium sorbate, sodium benzoate, and sodium lactate and sodium diacetate.

II. Materials and Methods

A. Bacterial Isolates

Listeria monocytogenes strains 101 and 108 and *Salmonella* Typhimurium DT104 strains 2380 and 2486 (animal isolates originally isolated from animal sources by D. Hancock and T. Besser, College of Veterinary Medicine, Washington State University, Pullman, WA) were obtained from the University of Tennessee, Department of Food Science and Technology culture collection. *L. monocytogenes* and *S.* Typhimurium were grown in trypic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) with 0.6% yeast extract (Difco), TSBYE, at 32°C. Agar medium for *L. monocytogenes* and *S.* Typhimurium as tryptic soy agar (TSA; Difco). Cultures were maintained on TSA slants at 4°C and transferred monthly to maintain viability. A working culture was prepared by inoculating a loopful of culture into 9 ml of TSB. The culture was then subjected to two successive 24 h transfers before being used.

B. Food Preservative Antimicrobials

Potassium sorbate (Acros Organics, Geel, Belgium), sodium benzoate (Sigma-Aldrich Chemicals, St.Louis, MO), sodium diacetate (Sigma-Aldrich Chemicals) and sodium lactate (Purac America, Lincolnshire, IL) were used. Stock solutions were prepared with 25 g of each antimicrobial in 100 ml of deionized water. Stock solutions were mixed thoroughly and then filter-sterilized using 0.45-μm-membrane filter (Millipore, Bedford, MA.). Fresh stock solutions were prepared weekly and stored at 4°C until use.

C. Agar Dilution Assay

An agar dilution assay was performed to adapt strains to antimicrobials used. *L. monocytogenes* and *S.* Typhimurium isolates were exposed to food preservative antimicrobials by spreading 0.5 μl from a 24 h culture (8-9 log CFU/ml) onto15 ml trypticase soy agar (TSA; Difco). Petri plates were prepared to containing a range of increasing antimicrobial concentrations. Plates were incubated at 32°C for 48 h. Potassium sorbate and sodium benzoate were in the range of 0.1-0.4% (w/v), sodium lactate at 1.0-4.0% (w/v), and sodium diacetate 0.5%-2.0% (w/v). The pH of the media was adjusted to 6.0 with sterile 1N HCl before pouring plates. Growth observed was noted as heavy, moderate, visible, hazy or no growth. Minimum inhibitory concentrations (MICs) were defined as the lowest concentration at which growth was completed inhibited after 48 h. Hazy or no growth was considered the limit of growth for the culture at a particular concentration of antimicrobial. When microbial growth occurred, cells were re-grown on TSA slants in the presence of that concentration and incubated at 32°C for 48 h. These strains were considered "adapted" or "resistant" to the highest antimicrobial concentration used. Cultures were maintained on tryptic soy agar (TSA) (Difco) slants with the highest concentration at 4°C for further analysis.

D. Comparison of Growth of Adapted and Non-Adapted Strains

Growth curves were carried out to compare adapted and non-adapted (parent) strains at the same concentration of antimicrobial and in the presence and absence of glucose in the media. Strains of *Listeria monocygenes* and *Salmonella* Typhimurium previously adapted to 0.4% (w/v) potassium sorbate (PS) and sodium benzoate (SB), 4.0% (w/v)

sodium lactate (SL) and sodium diacetate (SD) 0.5% (w/v). Two media were used, tryptic soy broth (TSBG, Difco) which contains 0.25% glucose and TSB with no glucose. The purpose of using two different media was to determine if the presence of glucose affected growth and adaptation to food preservatives antimicrobials.

A loopful (10μl) of previously adapted strains from the agar dilution assay was used in the broth dilution assay. Adapted and non-adapted (parent) strains were both exposed to the same concentration of antimicrobial in TSBG and TSB, both adjusted to 6.0 with sterile 1N HCl. Tubes were incubated at 32°C for 24-48 h or until turbidity was detectable. One ml of each tube was then transferred to another tube containing the same media and same concentration of antimicrobial. Tubes were incubated at 32°C for 24-48 h or until turbidity was detectable. Cultures were then inoculated into the same medium at higher concentrations, tubes were incubated for 24 h and the number of organism was enumerated at 0, 3, 6, 12 and 24 h using an automatic spiral plater (Don Whitley Scientific, West Yorkshire, England). Plates were incubated at 32°C for 48 h and the number of colonies read using a Protocol automatic reader (Synoptics Limited, Cambridge, UK). The pH of each strain grown in TSBG and TSB was measured at 0, 6, 12 and 24 h using an Accumet pH meter (Fisher Scientific, St. Louis, MO) to determine if the presence of glucose caused a reduction in the pH of the media by the microorganisms that could affect tolerance to the antimicrobials.

III. Results

An agar dilution type assay was used to adapt cells gradually to increasing concentrations of each antimicrobial. *Listeria* and *Salmonella* strains were adapted to 0.4% potassium sorbate (Table 1.0) and 0.4% for sodium benzoate (Table 2.0), 4.0% sodium lactate (Table 3.0) and 1.0% sodium diacetate (Table 4.0). Cells grown at the highest concentration were considered "adapted" or "resistant" strains.

Growth of adapted strains was compared to parent strains at the same concentrations. TSBG and TSB were used to evaluate if the presence of glucose affected adaptation and susceptibility of adapted and non-adapted strains. Most adapted *Listeria* strains were less susceptible and had higher growth yield than non-adapted strains when exposed to same concentration and grown TSBG or TSB. *L. monocytogenes* strain 101 adapted to 0.4% PS grown in TSBG has ca.0.6 log higher growth than the non-adapted strain after 24 h (Appendix 1A). *L. monocytogenes* strain 108 adapted to 0.4% PS grown in TSBG showed better growth by 1 log unit after 6 h than non-adapted strain but reached similar growth as non-adapted after 24 h (Appendix 1B). Adapted *L. monocytogenes* strain 101 to 0.4% SB in TSBG showed by 2 log higher growth level than non-adapted parent strain after 24 h (Appendix 5A). Benzoate-adapted *L. monocytogenes* strain 108 had approximately a 1 log higher growth level than non-adapted strains after 24 h (Appendix 5B).

Table 1.0 Growth of *Listeria monocytogenes* and *Salmonella* Typhimurium strains to increasing concentrations of potassium sorbate (PS) at pH 6.0 in 48 h.

Strains	**Potassium Sorbate %**						
	0.1	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**MIC[a]**
L. monocytogenes						n/a	
101	+++	+++	+	+	+		0.5%
						n/a	
108	+++	+++	++	+/-	+		0.5%
						n/a	
310	+++	+++	++	++	+		0.5%
						n/a	
Scott A	+++	+++	++	++	+		0.5 %
S. Typhimurium							
2380	+++	+++	+++	+++	+	+/-	0.5%
2486	+++	+++	+++	+++	++	+	0.6%
2576	+++	+++	+++	+++	++	+	0.6%
2582	+++	+++	+++	+++	++	+	0.6%

+++ Heavy, ++Moderate, +Visible, +/-Hazy, -Negative n/a no exposure
[a] Minimum inhibitory concentration

Table 2.0 Growth of *L. monocytogenes* and *S.* Typhimurium strains to increasing concentrations of sodium benzoate (SB) at pH 6.0 in 48 h.

Strains	**Sodium Benzoate %**						
	0.1	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**MIC[a]**
L. monocytogenes							
101	+++	+++	+	+	-	n/a	0.4%
108	+++	+++	++	+/-	-	n/a	0.3%
310	+++	+++	++	++	-	n/a	0.4%
Scott A	+++	+++	++	++	+/-	n/a	0.4 %
S. Typhimurium							
2380	+++	+++	+++	++	+	+/-	0.5%
2486	+++	+++	+++	+++	+	-	0.5%
2576	+++	+++	+++	+++	++	-	0.5%
2582	+++	+++	+++	+++	+	+/-	0.5%

+++ Heavy, ++Moderate, +Visible, +/-Hazy, -Negative n/a no exposure
[a] Minimum inhibitory concentration

Table 3.0 Growth of *Listeria monocytogenes* and *S.* Typhimurium strains to increasing concentrations of sodium lactate (SL) at pH 6.0 in 48 h.

Strains	**Sodium Lactate %**				
	1.0	**2.0**	**3.0**	**4.0**	**MIC[a]**
L. monocytogenes					
101	+++	+++	+++	+++	4.0%
108	+++	+++	+++	+++	4.0%
310	+++	+++	+++	+++	4.0%
Scott A	+++	+++	+++	+++	4.0%
S. Typhimurium					
2380	+++	+++	+++	+++	4.0%
2486	+++	+++	+++	+++	4.0%
2576	+++	+++	+++	+++	4.0%
2582	+++	+++	+++	+++	4.0%

+++ Heavy, ++Moderate, +Visible, +/-Hazy, -Negative n/a no exposure
[a] Minimum inhibitory concentration

Table 4.0 Growth of *L. monocytogenes* and *S.* Typhimurium strains to increasing concentrations of sodium diacetate (SD) at pH 6.0 in 48 h.

Strains	**Sodium Diacetate %**			
	0.5	**1.0**	**2.0**	**MIC[a]**
L. monocytogenes	+++	+	-	
101				1.0%
	+++	+	-	
				1.0%
108				
	++	+	-	
310				1.0%
	++	++	-	
Scott A				1.0%
S. Typhimurium				
	+++	+	-	
2380				1.0%
	+++	+++	-	
2486				1.0%
	+++	+++	-	
2576				1.0%
	+++	+/-	-	
2582				1.0%

+++ Heavy, ++Moderate, +Visible, +/-Hazy, -Negative
[a] Minimum inhibitory concentration

L. monocytogenes 101 and108 adapted to 4.0% SL grown in TSBG showed slightly better growth (≥ 0.5 log CFU/ml) than the non-adapted strains for the first 6 h but reached similar levels (log 6.5 and log 8.5, respectively) as the non-adapted strains at 24 h (Appendix 9A and 9B). *L. monocytogenes* strain 101 adapted to 0.5% SD had better growth (≥1.0 log CFU/ml) in the first 6 h then reached the same growth (log 7.5 CFU/ml) as non-adapted strains after 12 h (Appendix 13A). Adapted *L. monocytogenes* strain 108 to 0.5% SD in regular TSBG showed higher growth by 1.0 log unit than non-adapted after 24 h of (Appendix 13B).

S. Typhimurium strain 2380 adapted to 0.4% PS grown in TSBG showed higher growth by 1 log unit than the non-adapted strain after 24 h (Appendix 3A). Adapted *S.* Typhimurium 2486 adapted to 0.4% PS grown in TSBG had higher growth yield by 3 log than the non-adapted strain after 24 h (Appendix 3B). Strains *S.* Typhimurium 2380 and 2486 adapted to 0.4% SB grown in TSBG showed very similar reactions to that of potassium sorbate (Appendix 7A and 7B).

Non-adapted *S.* Typhimurium strain 2380 exposed to 0.4% PS and SB grown in TSBG were more susceptible than adapted strains showing a 4 log reduction in the first 6 h then reached the same level of growth as adapted strains after 12 h (Appendix 4.0 and 7.0). Non-adapted *S.* Typhimurium 2486 showed susceptibility to both antimicrobials and decreased by 4 log by 6 h (Appendix 5.0 and 7.0). Parent strain of *S.* Typhimurium 2380 and 2486 demonstrated at 1.5-2.0 log decrease in viable cells in the first 3 h of exposure to sodium lactate and sodium diacetate but increased in numbers thereafter (Appendix 11.0 and 15.0). Adapted *S.* Typhimurium strain generally grew at the same rate as

controls with the exception of the strain 2486 with sodium diacetate that showed an initial increase (Appendix 15B).

When all *Listeria* and *Salmonella* strains were grown in TSB with no glucose (TSB), adapted strains became more susceptible to all antimicrobials but still showed slightly better growth than non-adapted strains. Adapted *L. monocytogenes* 101 and 108 adapted to 0.4% PS and SB grown in TSB showed slightly better growth (>0.5 log CFU/ml) for the first 12 h but reached similar levels of ~6.5 log CFU/ml as non-adapted cells by 24 h (Appendixes 1.0). A comparison of media, adapted and non-adapted *L. monocytogenes* strains 101 to 0.4% PS and BS, showed at least 1 log unit increased when grown in TSBG compared when grown in TSB (Appendix 1A and 3A). To the contrary, adapted and non-adapted *L. monocytogenes* strains 108 to 0.4% PS and BS seemed not to be affected by the absence of glucose in the growth media (Appendix 1B and 3B) since both strains reached similar growth after 24 h. Adapted *L. monocytogenes* strains 101 and 108 to 4.0% SL grown in TSB showed better growth by 0.5 log units first 12 h but reached similar growth (~7.5 log CFU/ml) as non-adapted strains after 24 h (Appendix 9A and 9B). A comparison of media, adapted and non-adapted *L. monocytogenes* strains 101and 108 to 4.0% SL, showed at least 1 log unit increased when grown in TSBG compared when grown in TSB (Appendix 9A and 9B). Adapted *L. monocytogenes* strains 101 and 108 to 0.5% SD grown in TSB showed better growth by 1.0 log unit than non-adapted strains after 24 h (Appendix 13A and 13B). For adapted and non-adapted *L. monocytogenes* strains 101 to 0.5% SD, there was at least 2 log units increased when grown in regular TSB compared when grown in TSBG compared when grown in TSB

(Appendix 13A). To the contrary, adapted and non-adapted *L. monocytogenes* strains 108 to 0.5% SD showed only 0.5 log units increased when grown in TSBG compared when grown in TSB (Appendix 13B).

Adapted *S.* Typhimurium strains 2380 and 2486 to 0.4% PS and SB grown in TSB showed highest growth yields by 3 log units than non-adapted strains after 24 h (Appendix 3.0 and 7.0) except non-adapted strain 2380 0.4% SB (Appendix 7A) that showed similar growth after 12 h as adapted strain. This clearly showed that *S.* Typhimurium adapted strains were able to resist and proliferate under the presence of the antimicrobial without any major growth reduction. Adapted *S.* Typhimurium strains 2380 and 2486 to 4.0% SL grown in TSB showed better growth first 6 h but reached similar growth as parent strains after 24 h (Appendix 11.0 and 13.0). A comparison of media indicated no major differences for adapted *S.* Typhimurium strains 2380 and 2486 to 0.4% PS when grown in TSBG or TSB (Appendix 3.0). To the contrary, adapted *S.* Typhimurium strains 2380 and 2486 to 0.4% SB showed at least 3 log units for strain 2380 and 1 log unit for strain 2486 more when grown in TSB than when grown TSBG (Appendix 7.0). Non-adapted *S.* Typhimurium strains 2380 and 2486 to 0.4% PS and BS showed susceptibility to both antimicrobials when grown in either TSBG or TSB except strain 2380 that showed high growth yield (8.0 log CFU/ml) in TSB (Appendix 3.0 and 7.0). *S.* Typhimurium non-adapted strain 2380 exposed to 4.0% SL grown in TSB showed better growth by one log unit first 12 h than adapted strain but then decreased growth by 4 log units after 24 h (Appendix 11.0). Adapted *S.* Typhimurium strains 2380 and 2486 to 0.5% SD grown in TSB showed higher growth by at least 2 log units than

non-adapted strains after 24 h (Appendix 13.0). *S.* Typhimurium non-adapted strains exposed to 0.5% SD grown in TSB showed susceptibility and reduction in growth after 24 h (Appendix 13.0). A comparison of media indicated major differences in growth and susceptibility between adapted and non-adapted *S.* Typhimurium strain 2380 to 0.4% SL and 0.5% SD when grown in TSBG or TSB w (Appendix 11.0 and 13.0). Adapted *S.* Typhimurium strain 2380 to 4.0% SL grown in TSBG showed at least 1 log unit higher than strains grown in TSB (Appendix 11A). Adapted and non-adapted *S.* Typhimurium strain 2380 to 0.5% SD grown in TSBG showed at least 4 log units higher for non-adapted and 1 log unit higher for adapted than strains grown in TSB (Appendix 13A). Non adapted *S.* Typhimurium strain 2380 to 4.0% SL grown in TSBG showed at least 4 log units higher than non adapted strains grown in TSB (Appendix 11A). Interestingly, adapted and non-adapted *S.* Typhimurium strain 2486 to 4.0% SL grown in TSBG showed at least 1 log unit lower than strains grown in TSB (Appendix 11B). There were no major differences between adapted and non-adapted *S.* Typhimurium strain 2486 to 0.5% SD grown in TSBG and TSB (Appendix 15B).

Adapted and parent strains were exposed to higher concentrations of each antimicrobial and grown in TSBG and TSB (Appendix 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 and 16.0). Adapted *L. monocytogenes* strains 101 and 108 exposed to 0.5% PS and SB grown in TSBG showed slightly better growth (>0.5 log CFU/ml) than non-adapted strains after 24 h (Appendix 2.0 and 4.0). Adapted *L. monocytogenes* strains 101 and 108 exposed to 5.0% SL and 1.0% SD grown in TSBG showed higher growth by 2 log than non-adapted strains after 24 h (Appendix 6.0 and 8.0) .Non-adapted *L. monocytogenes*

strains exposed to higher concentrations of SD (1.0%) and SL (5.0%) grown in TSBG showed higher susceptibility by 3 log reduction after 24 h compared to adapted strains (Appendix 6.0 and 8.0). All adapted *S.* Typhimurium strains 2380 and 2486 grown in TSBG showed higher growth yield by 3 log than non-adapted strains after 24 h (Appendix 10.0, 12.0, 14.0 and 16.0)) except parent strain 2380 exposed to 1.0% SD that showed similar growth (5.0 log CFU/ml) as parent after 12 h of exposure (Appendix 16A).

Adapted strains became more susceptible to all antimicrobials when grown in TSB but still showed slightly better growth than non-adapted strains (Appendix 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 and 16.0). Adapted *L. monocytogenes* strains 101 and 108 exposed to 0.5% PS and SB grown in TSB showed higher growth yield by 1 to 2 log units than non-adapted strains first 12 h but reached similar growth after 24 (Appendix 2.0 and 6.0). Adapted *L. monocytogenes* strains 101 and 108 exposed to 5.0% SL grown in TSB showed higher growth yield by 2-4 log units than non-adapted strains after 24 h (Appendix 10.0). Adapted *L. monocytogenes* strains 101 and 108 exposed to 1.0% SD grown in TSB showed growth reduction by 4 log units as non-adapted strains after 24 h of incubation (Appendix 14.0). All adapted *S.* Typhimurium strains 2380 and 2486 to higher concentrations of all antimicrobials tested and grown in TSB showed better growth by 3 to 4 log units than non-adapted strains after 24 h (Appendix 4.0, 8.0, 12.0 and 16.0) except adapted strains 2380 and 2486 to 1.0% SD that showed similar growth reduction (3-4 log CFU/ml) as non-adapted strain (Appendix 16.0).

The pH of each strain grown in TSBG and TSB was measured at 0, 6, 12 and 24 h to determine if the presence of glucose caused a reduction in the pH of the media by the microorganisms that could affect tolerance to the antimicrobials (Appendix 17.0-14.0). Most *Listeria monocytogenes* and *Salmonella* Typhimurium strains grown in TSBG with after 24 h had pHs between 5.0-5.5. This was expected due to glucose fermentation by the microorganisms that lowered the media pH and made the organism more tolerant to the antimicrobial. The only antimicrobial that lowered the pH to 4.8-5.0 was sodium diacetate for all strains (Appendix 20.0 and 24.0). The pHs of all *Listeria* and *Salmonella* strains grown in TSB were around 5.8-6.0, which was similar to the original adjusted media pH of 6.0. The only strains that showed lower pHs between 5.5.-5.8 were *Salmonella* strain 2380 and 2486 adapted to PS and BS (Appendix 21.0 and 22.0).

IV. Discussion

When microorganisms are stressed, an adaptive or protective response may follow to protect the cell. Microorganisms respond to stress by increasing their tolerance to the same stress, another type of stress and even to subsequent harsher stress. Most bacteria have an inherent tolerance level to a particular stress but still a transient or adaptive tolerance may be induced (Yousef and Courtney, 2003). Adaptation enhances tolerance to environmental, chemical and biological stresses and may promote survival or growth in adverse environments (Johnson, 2003). Resistance and adaptation to antimicrobials may vary depending on the physiological status of the organism, the type and concentration of antimicrobial and the physicochemical characteristics of the external environment. All these factors will have an impact on the ability of organism to proliferate and survive in normally adverse and even harsher environments.

In this study it was found that by adapting *Listeria monocytogenes* and *Salmonella* Typhimurium strains to certain concentrations of traditional food antimicrobials, adapted strains had higher growth and were less susceptible than most non-adapted strains when exposed to high concentrations. It is known that microorganisms may develop resistance to antimicrobial agents such as weak organic acids upon subsequent applications (Lueck, 1980; Brul and Coote, 1999; Lin et al., 1996; Davis et al., 1996). The ability of *Listeria* and *Salmonella* cells to proliferate and survive subsequent exposures of antimicrobial compounds may be mediated by multiple mechanisms and/or resistance determinants in the bacterial cell. Some cells will grow and survive after antimicrobial exposure because they may possess a degree of natural resistance (i.e. altered permeability, efflux pumps),

or may acquire it later through mutation or genetic exchange (Bower and Daeschel, 1999). Gould (1989) reported that vegetative bacterial cells exposed to environmental stresses adapted to the stresses in a variety of ways to maintain a homeostatic condition. These adaptations included activation and expression of new groups of genes that produce proteins that repair damage, maintain the cell or eliminate the stress. Rowbury (1998) suggested another way bacteria respond to stress was by secreted extracellular components that could be involved in inducing resistance responses by effectively warning bacteria of impending stress.

Once cells were adapted by using an agar dilution assay, adapted cells showed no susceptibility when grown again under same high concentration using a broth dilution assay. This once again confirmed the ability of both organisms to tolerate subsequent exposures of previous highest antimicrobials concentrations. Also, this acquired adaptation to all four antimicrobials was maintained for several weeks when adapted cells were conserved at 4°C in TSA slants (not shown). It was interesting to find that *L. monocytogenes,* a Gram-positive organism, was not necessarily more susceptible to most antimicrobials than *S.* Typhimurium. This was interesting because other studies have shown that Gram-negative bacteria are in general, more resistant to antimicrobials compounds due to the presence of the outer membrane (Hogan and Kolter, 2002). The precise reason behind this observation is not clear, but as other authors have suggested resistance of Gram positive to antimicrobial compounds is mostly related to mechanisms involving destruction or inactivation of toxic compounds, changes in the target site, or active efflux of the chemicals out of the cell (Russell, 2000). In addition, it was

demonstrated in this study that a multiple antibiotic resistant pathogen such as *Salmonella* Typhimurium DT104 was not necessarily more resistant to regulatory approved food antimicrobials.

The presence of glucose in the media also had an effect on cell growth and tolerance to most antimicrobials. It is known that adaptation to weak organic acids can be achieved in a medium supplemented with an adequate concentration of fermentable carbohydrate, such as glucose, to reduce the pH during growth (Buchanan and Eldelson, 1996; Samelis et al., 2002). The presence of sugar in the media promotes production of acid with subsequent acid habituation (Johnson, 2003). This was evident in most adapted strains that showed better growth than parent strains when grown in regular TSB that contained 0.25% glucose and had lower pHs. Wilde et al., (2000) showed that *Salmonella enterica* serovar Enteritidis were more resistant to heat and acid when grown in stationary phase cells in the presence of glucose compared to cells grown in the absence of an added carbon source. In the present study, this was evident on adapted strains where cells had higher growth the first 6-12 h when grown in regular TSB with glucose. The media pH was another factor that may have contributed to the antimicrobial tolerance. Cells become habituated to extreme harsh environmental conditions when incubated for brief periods at slightly acidic pH (Booth, 1985). Although it was also interesting to note that some adapted strains grown in TSB with no glucose still yield higher or same growth as adapted strains grown in TSB with no glucose. These findings still demonstrated that in not all instances, adaptation was not necessarily achieved by lower pH in the media; it

may have also been mediated by other resistance parameters in the bacterial cell such as altered permeability or efflux pumps activation.

Results of this study can vary greatly from results in a natural system. The unique environment of the food matrix may provide selective advantages to the pathogens and increase their tolerance to a particular condition (Davidson and Harrison, 2002). Actual exposure of foodborne pathogens in the food industry could be expected to be similar to the model used in this study. That is, if pathogenic microorganisms were re-exposed to an antimicrobial food preservative, it would be repeated exposure to the same or similar concentrations. Under these conditions, we found adapted *Listeria monocytogenes* and *Salmonella* Typhimurium cells to traditional regulatory-approved food antimicrobials resulted in higher yield counts and were less susceptible to subsequent exposure of same and higher concentrations than non-adapted strains. Even, these cells were not acid adapted or shocked, still there was some higher level of resistance and adaptation (not considering the presence of glucose in the medium). It is important to note that adapted or tolerant microorganisms would have to possess enhanced survival in order to survive in a food system. Parameters such as temperature, pH and water activity can have a big impact on the development of resistance to food antimicrobials. Again, this will depend on the physiological status of the organism, the type and concentration of antimicrobial and the physicochemical characteristics of the external environment.

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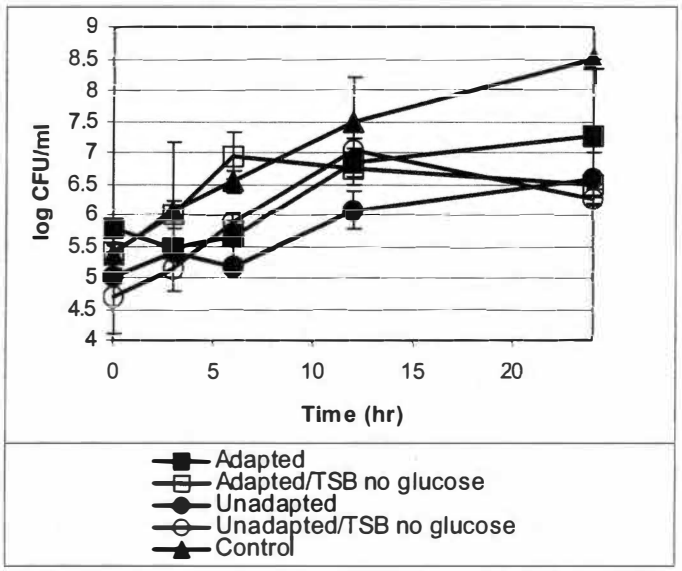
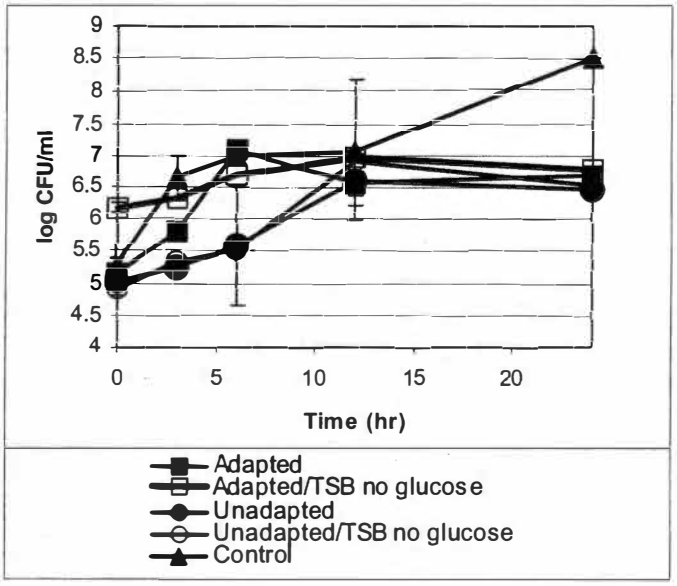
APPENDIX

Appendix 1.0: Adapted and unadapted *L. monocytogenes* 101 (A) and 108 (B) strains grown in 0.4% (w/v) potassium sorbate (PS) in TSB with and without glucose at pH 6.0 in 24 h.

A.

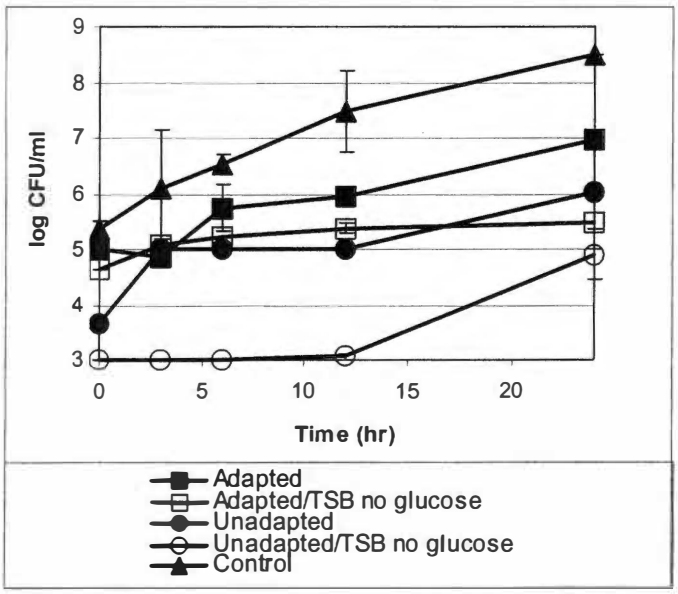


B.

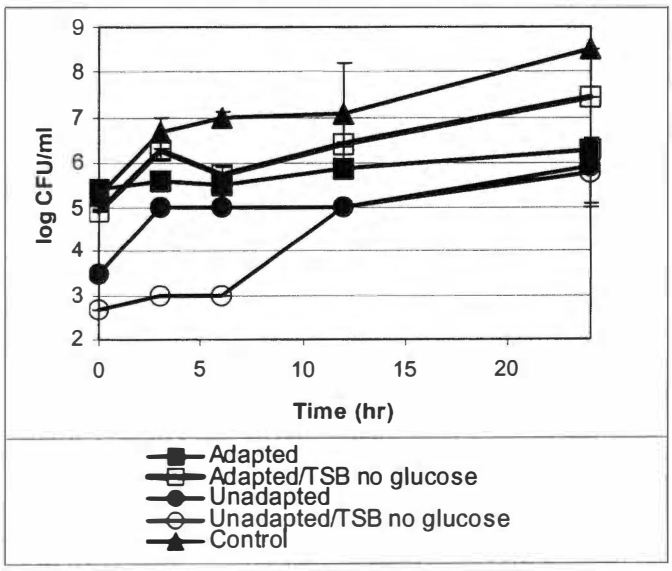


Appendix 2.0: Adapted and unadapted *L. monocytogenes* 101 (A) and 108 (B) strains grown in 0.5% (w/v) potassium sorbate (PS) in TSB with and without glucose at pH 6.0 in 24 h.

A.

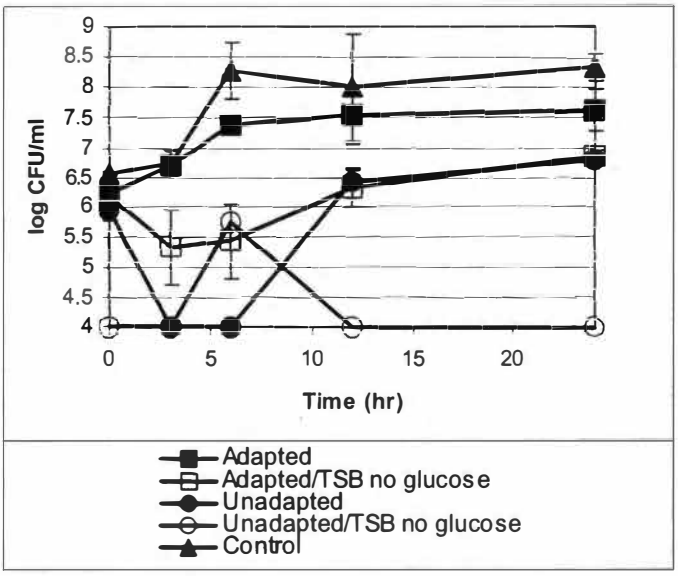


B.

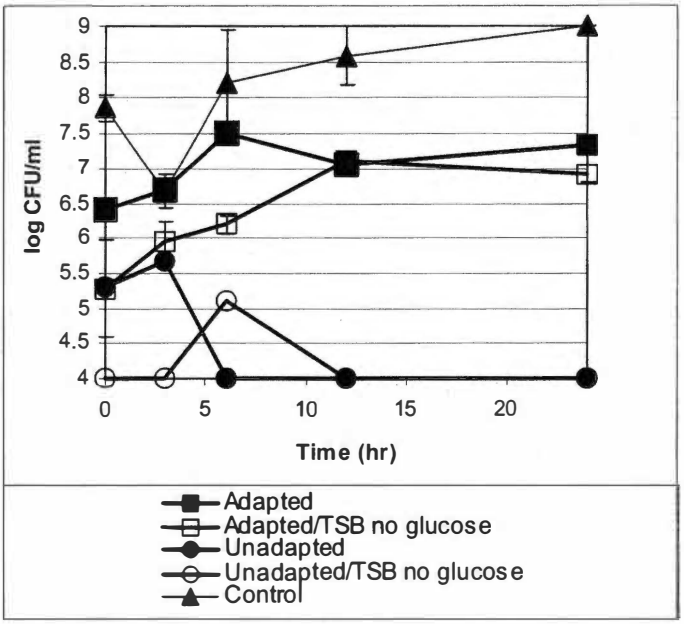


Appendix 3.0: Adapted and unadapted *S.* Typhimurium 2380 (A) and 2486 (B) strains grown in 0.4% (w/v) potassium sorbate (PS) in TSB with and without glucose at pH 6.0 in 24 h.

A.

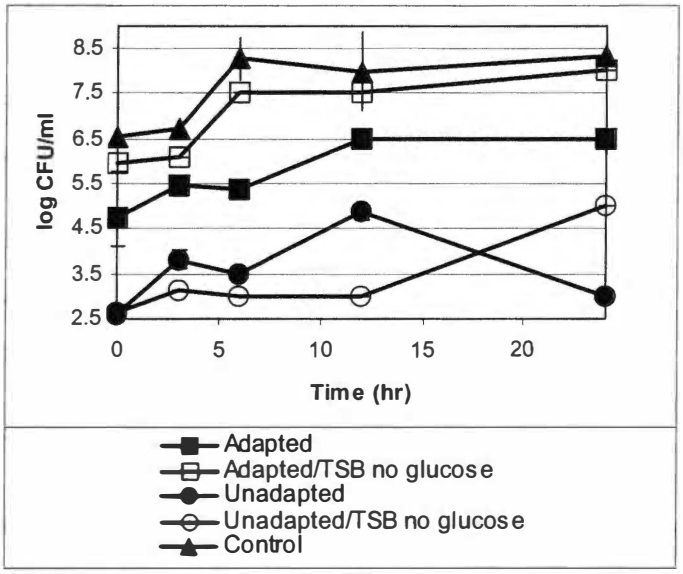


B.

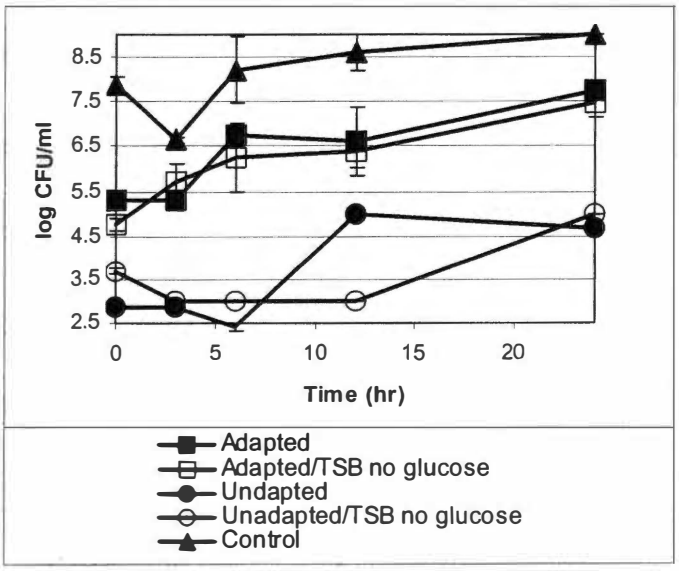


Appendix 4.0: Adapted and unadapted *S.* Typhimurium 2380 (A) and 2486 (B) strains grown in 0.5% (w/v) potassium sorbate (PS) in TSB with and without glucose at pH 6.0 in 24 h.

A.

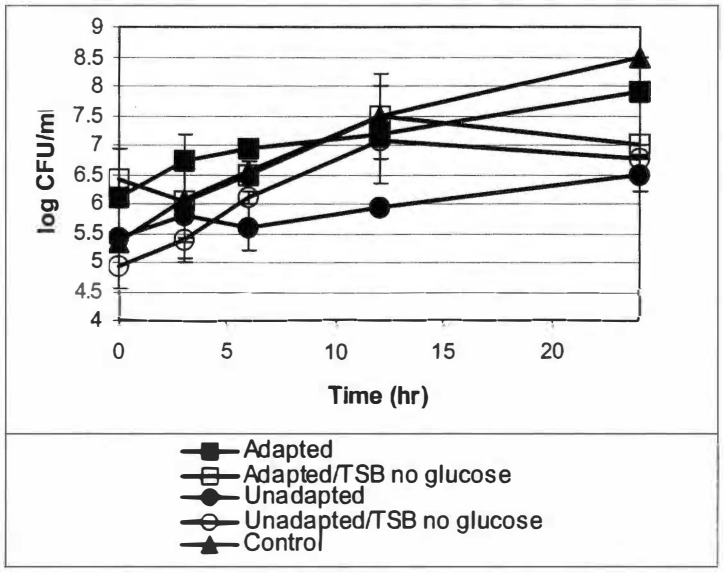


B.

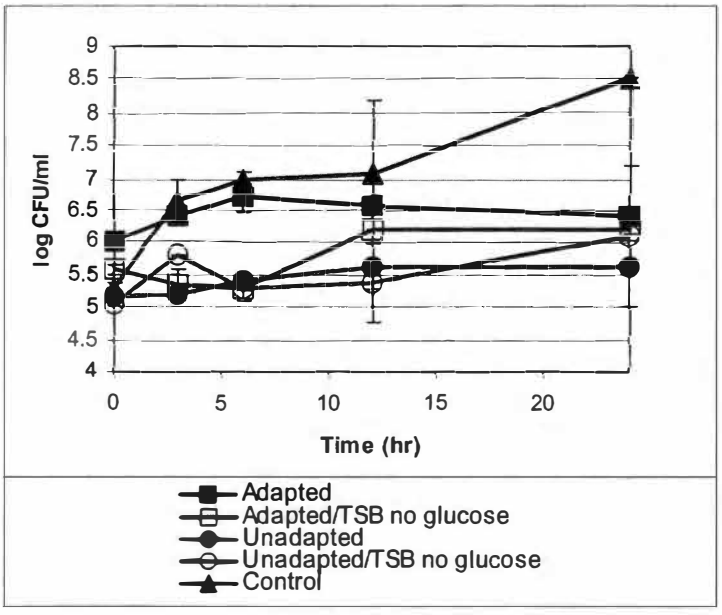


Appendix 5.0: Adapted and unadapted *L. monocytogenes* 101 (A) and 108 (B) strains grown in 0.4% (w/v) sodium benzoate (SB) in TSB with and without glucose at pH 6.0 in 24 h.

A.

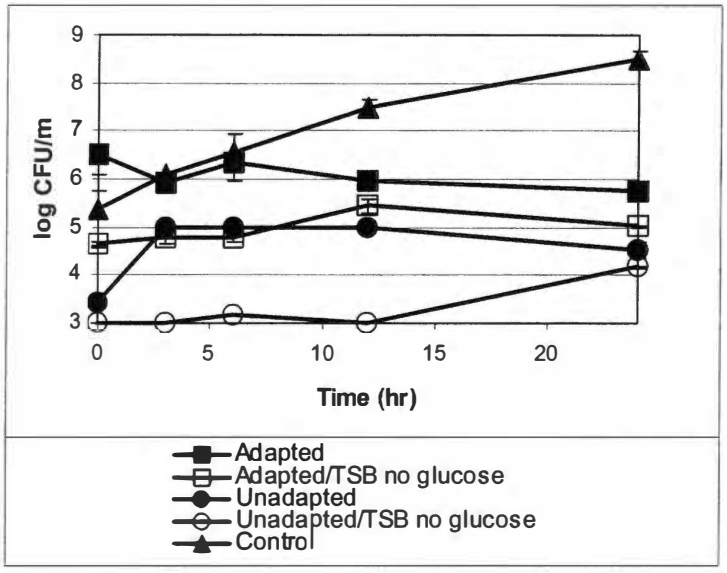


B.

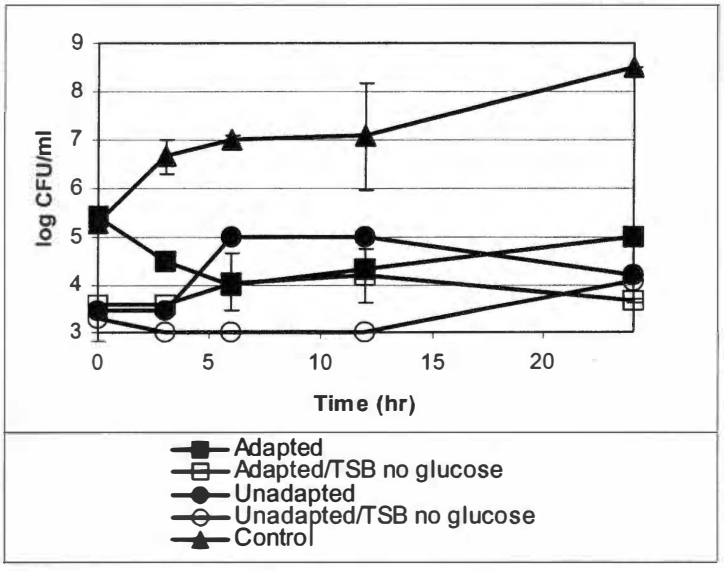


Appendix 6.0: Adapted and unadapted *L. monocytogenes* 101 (A) and 108 (B) strains grown in 0.5% (w/v) sodium benzoate (SB) in TSB with and without glucose at pH 6.0 in 24 h.

A.

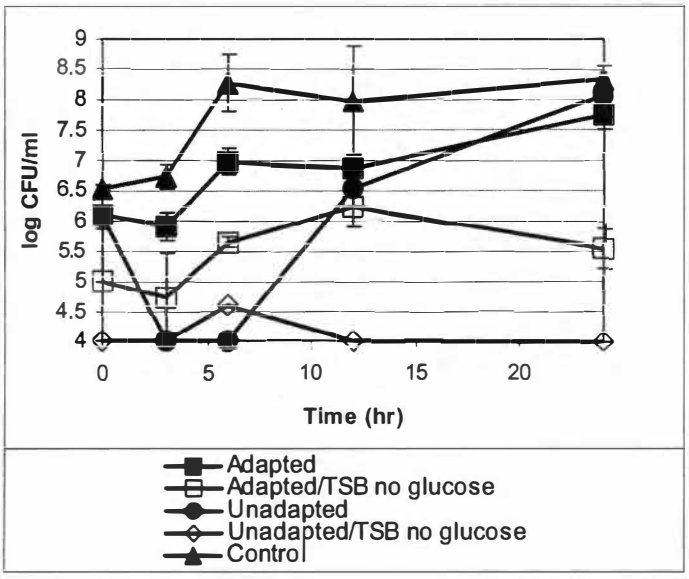


B.

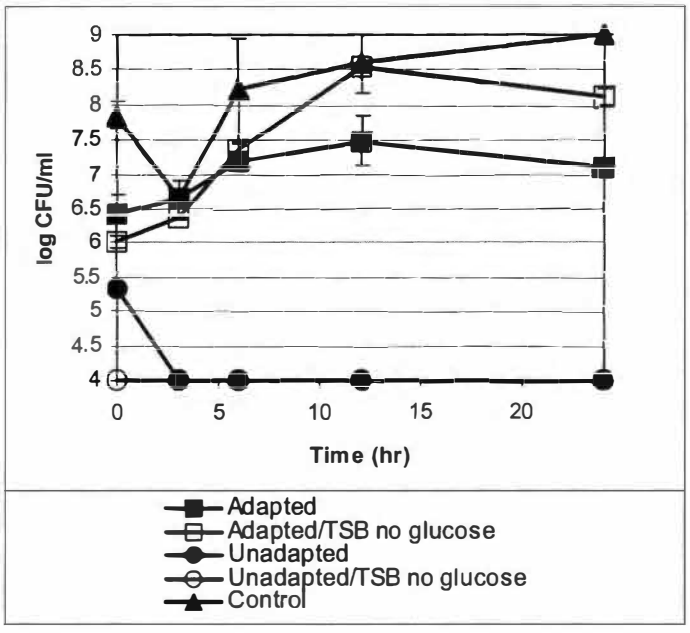


Appendix 7.0: Adapted and unadapted *S.* Typhimurium 2380 (A) and 2486 (B) strains grown in 0.4% (w/v) sodium benzoate (SB) in TSB with and without glucose at pH 6.0 in 24 h.

A.

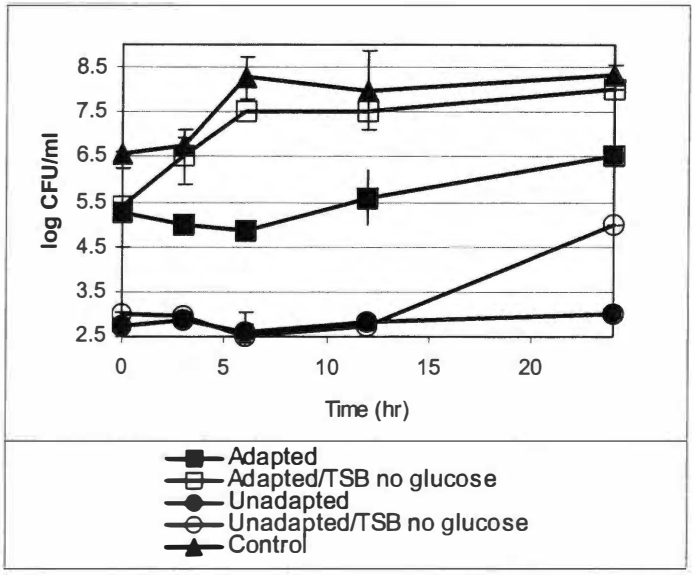


B.

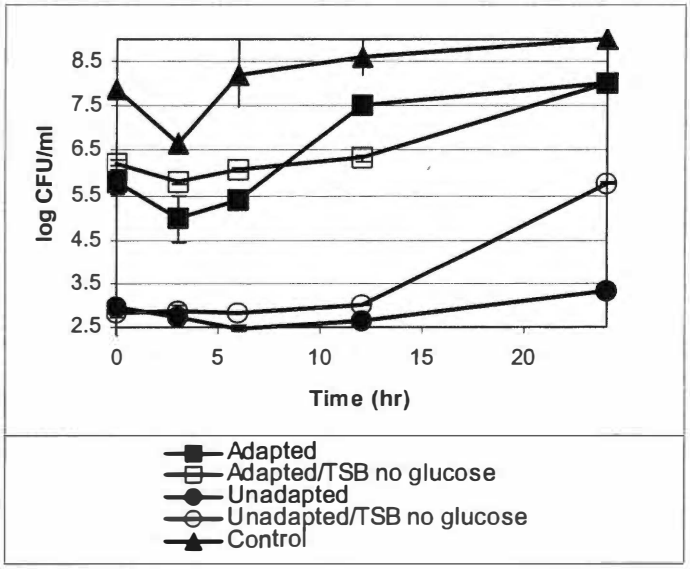


Appendix 8.0: Adapted and unadapted *S.* Typhimurium 2380 (A) and 2486 (B) strains grown in 0.5% (w/v) sodium benzoate (SB) in TSB with and without glucose at pH 6.0 in 24 h.

A.

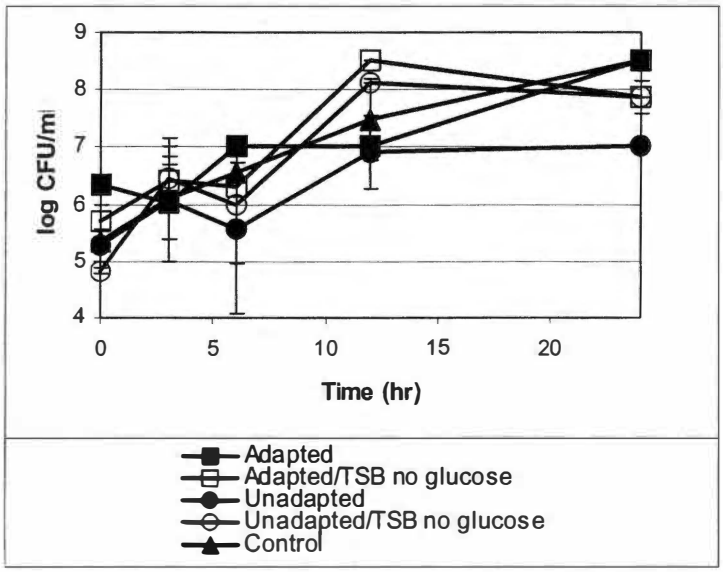


B.

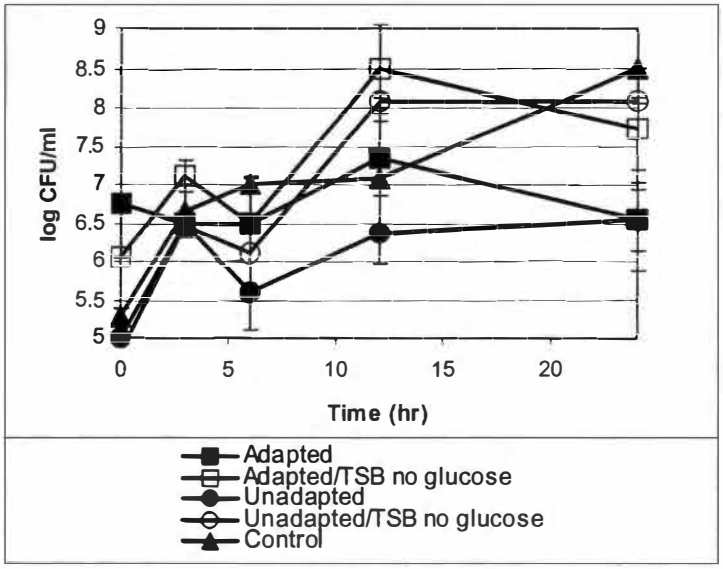


Appendix 9.0: Adapted and unadapted *L. monocytogenes* 101 (A) and 108 (B) strains grown in 4.0% (w/v) sodium lactate (SL) in TSB with and without glucose at pH 6.0 in 24 h.

A.

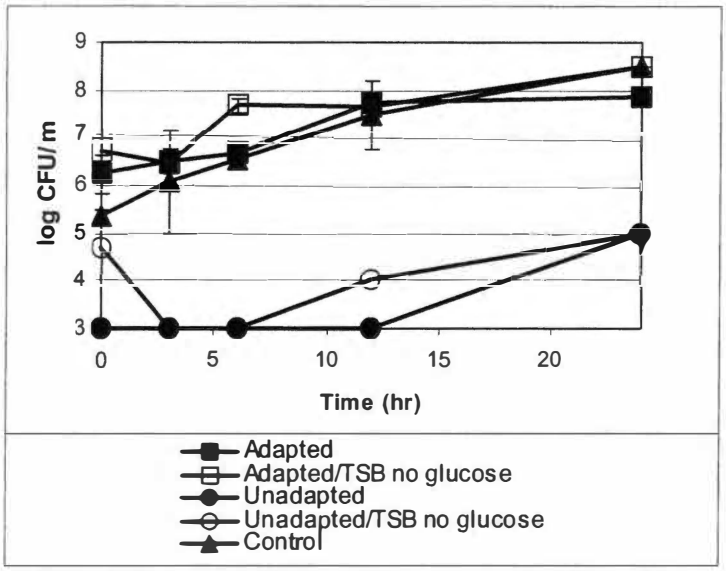


B.

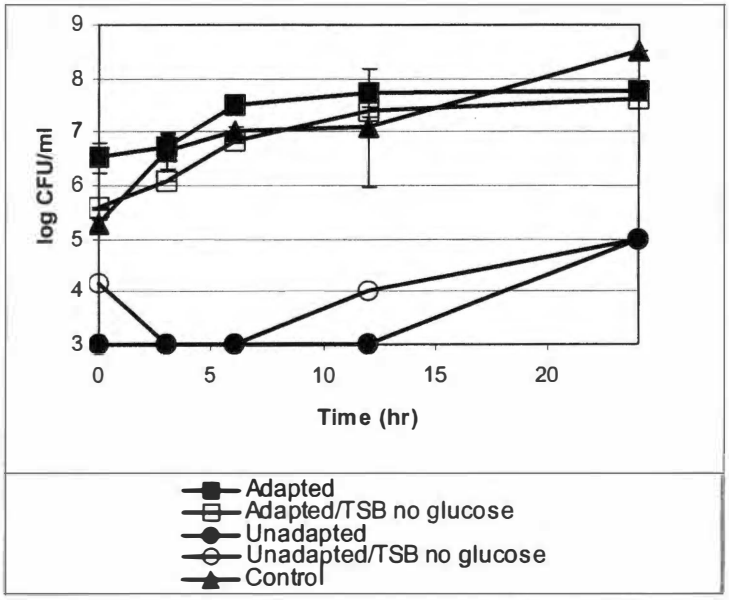


Appendix 10.0: Adapted and unadapted *L. monocytogenes* 101 (A) and 108 (B) strains grown in 5.0% (w/v) sodium lactate (SL) in TSB with and without glucose at pH 6.0 in 24 h.

A.

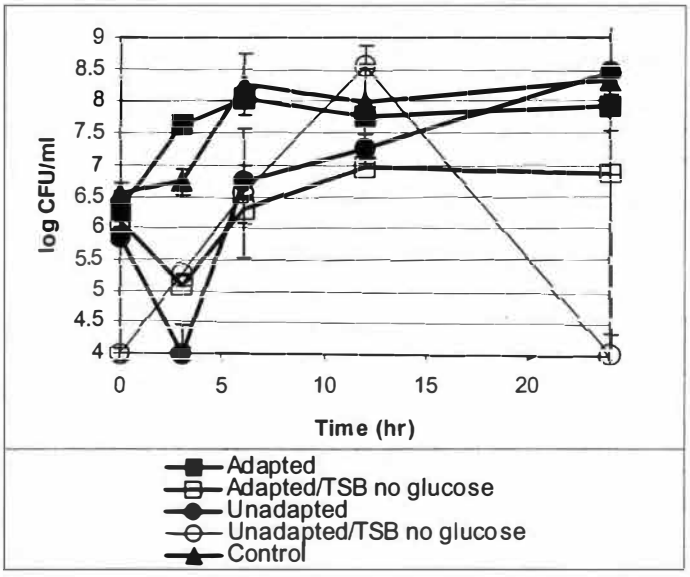


B.

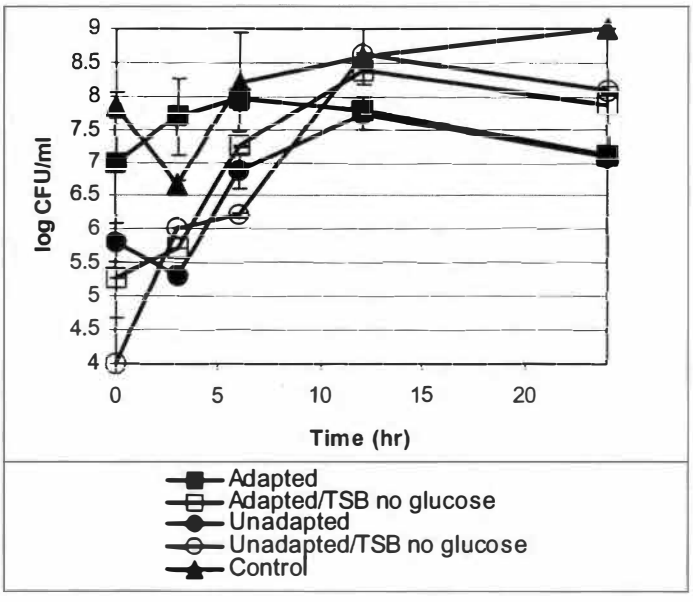


Appendix 11.0: Adapted and unadapted *S.* Typhimurium 2380 (A) and 2486 (B) strains grown in 4.0% (w/v) sodium lactate (SL) in TSB with and without glucose at pH 6.0 in 24 h.

A.

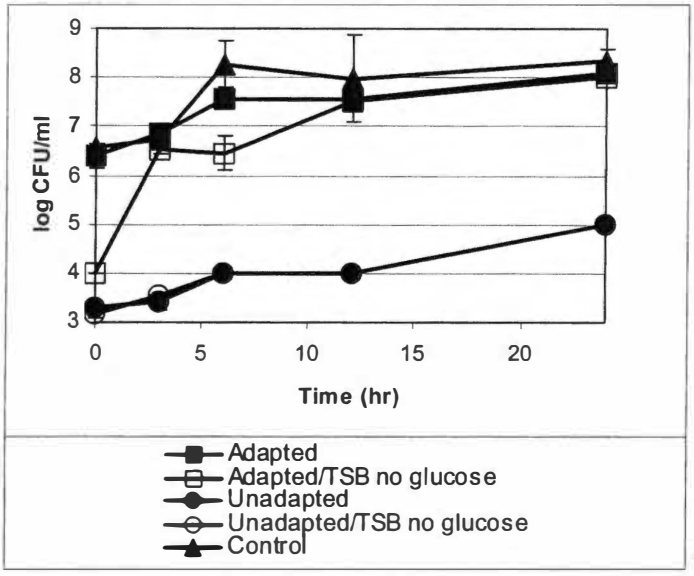


B.

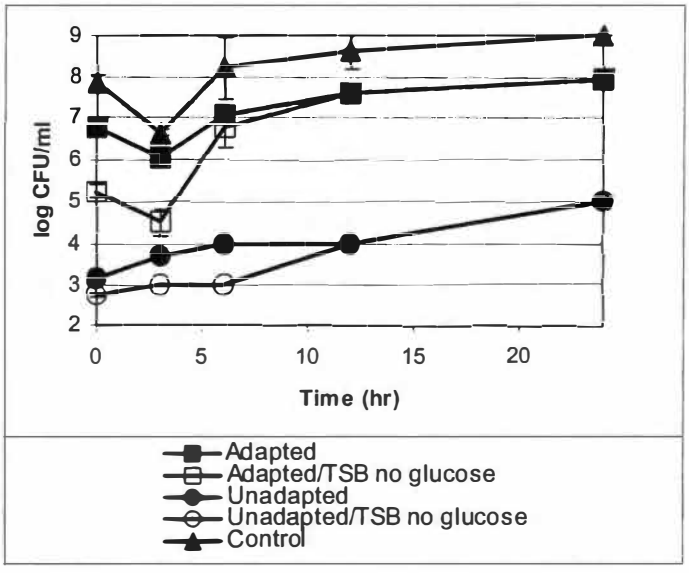


Appendix 12.0: Adapted and unadapted *S.* Typhimurium 2380 (A) and 2486 (B) strains grown in 5.0% (w/v) sodium lactate (SL) in TSB with and without glucose at pH 6.0 in 24 h.

A.

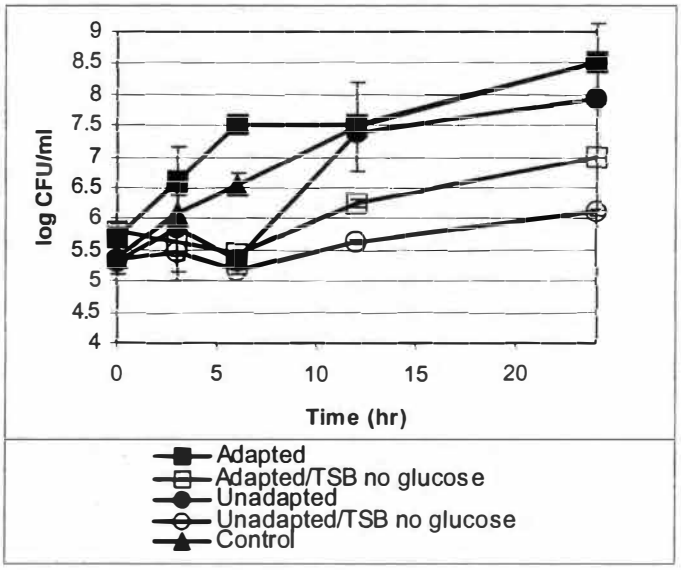


B.

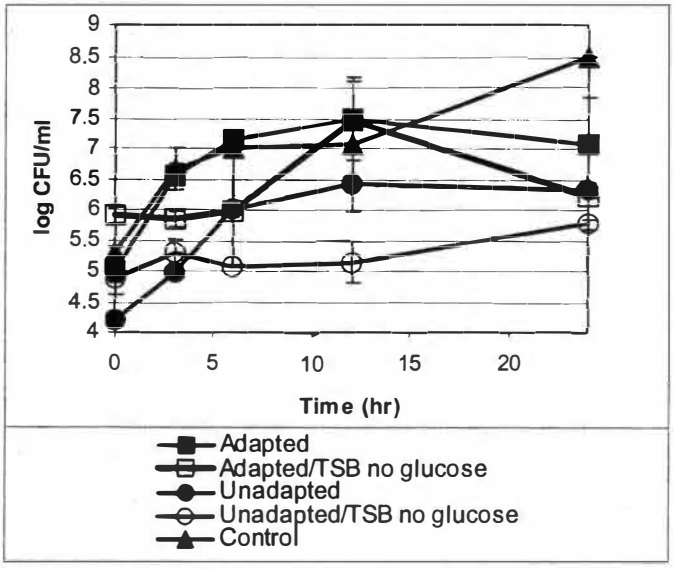


Appendix 13.0: Adapted and unadapted *L. monocytogenes* 101 (A) and 108 (B) strains grown in 0.5% (w/v) sodium diacetate (SD) in TSB with and without glucose at pH 6.0 in 24 h.

A.

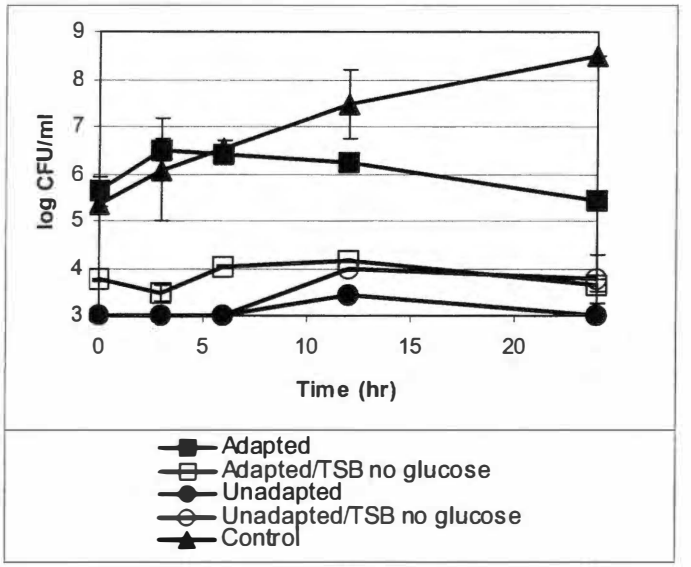


B.

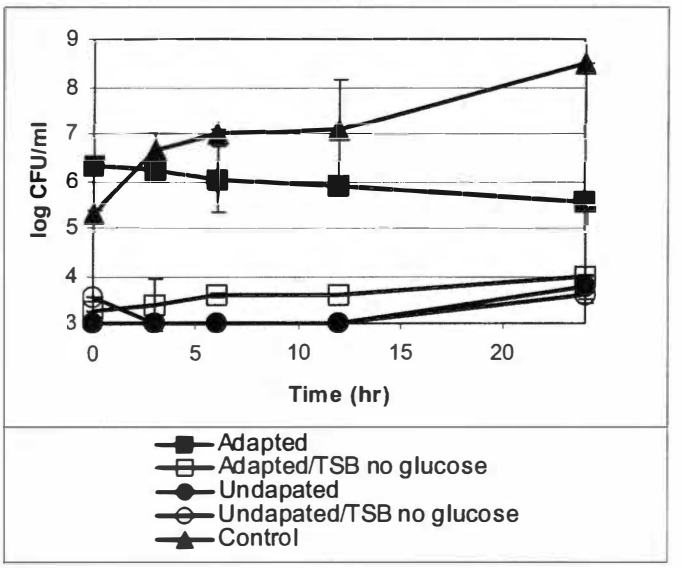


Appendix 14.0: Adapted and unadapted *L. monocytogenes* 101 (A) and 108 (B) strains grown in 1.0% (w/v) sodium diacetate (SD) in TSB with and without glucose at pH 6.0 in 24 h.

A.

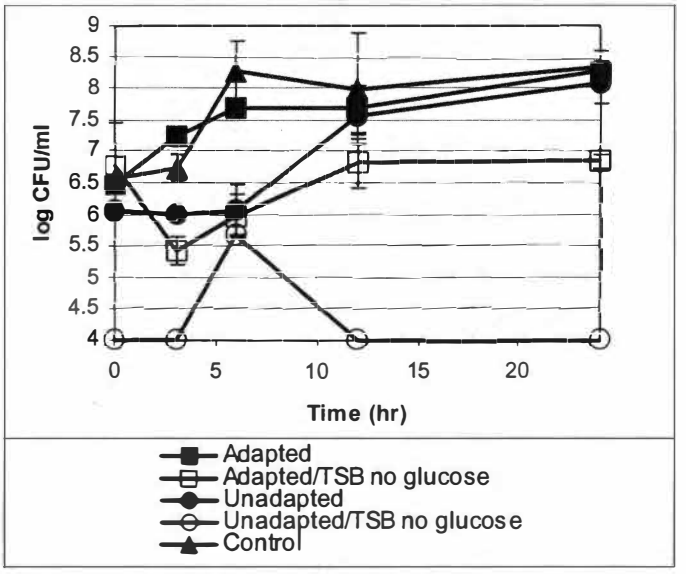


B.

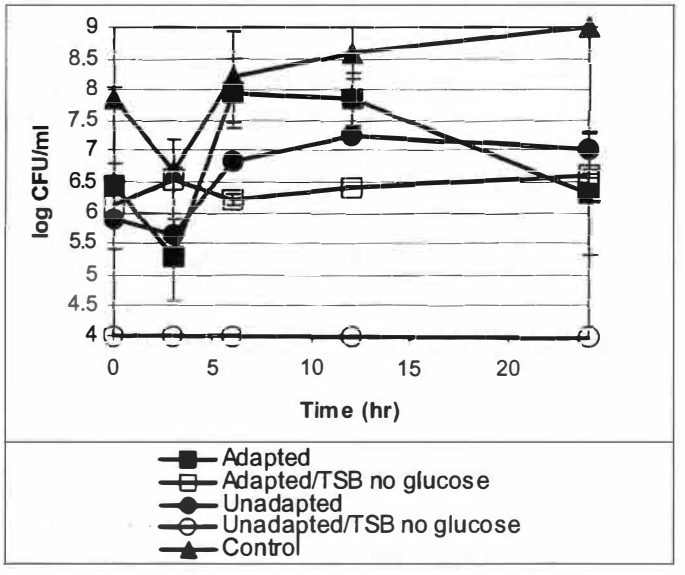


Appendix 15.0: Adapted and unadapted *S.* Typhimurium 2380 (A) and 2486 (B) strains grown in 0.5% (w/v) sodium diacetate (SD) in TSB with and without glucose at pH 6.0 in 24 h.

A.

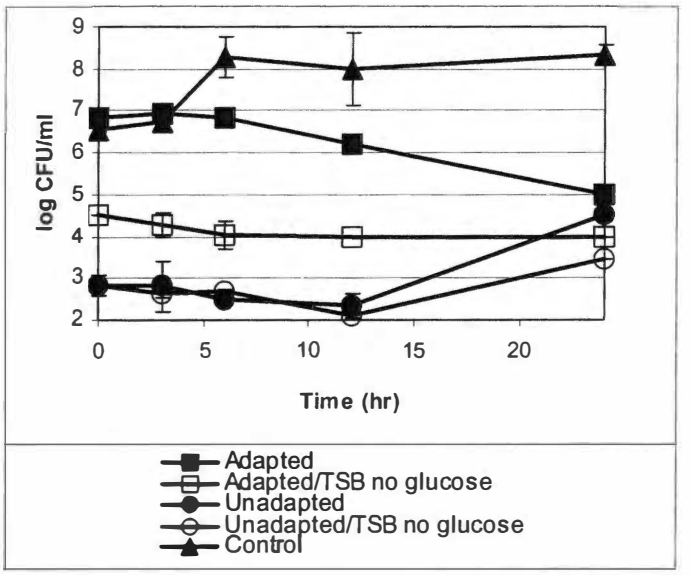


B.

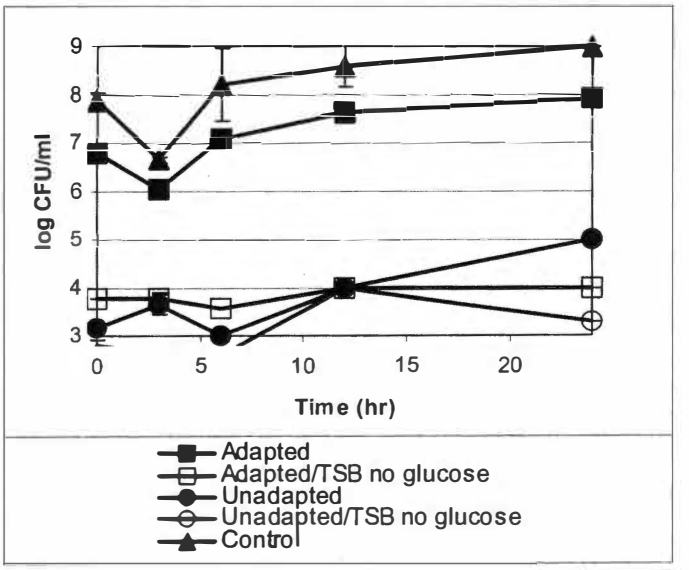


Appendix 16.0: Adapted and unadapted *S.* Typhimurium 2380 (A) and 2486 (B) strains grown in 1.0% (w/v) sodium diacetate (SD) in TSB with and without glucose at pH 6.0 in 24 h.

A.

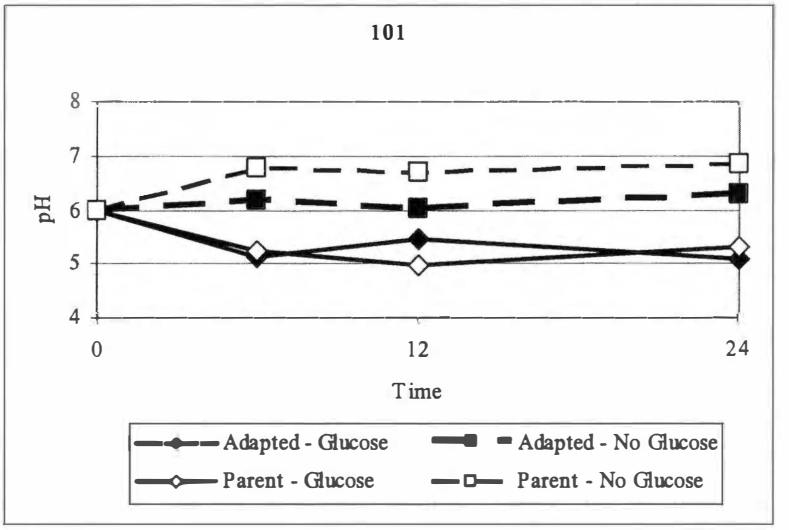


B.

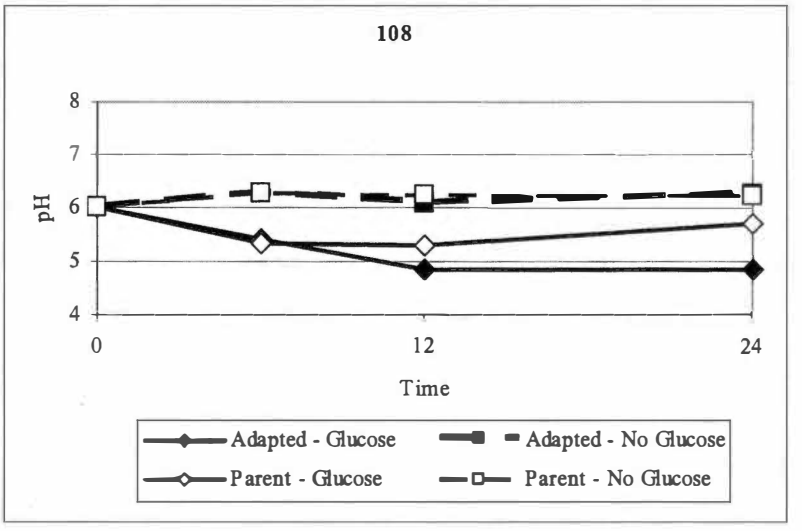


Appendix 17.0: pH measurements of adapted and parent *L. monocytogenes* 101 (A) and 108 (B) strains grown in 0.4% (w/v) potassium sorbate (PS) in TSB with and without glucose in 24 h.

A.

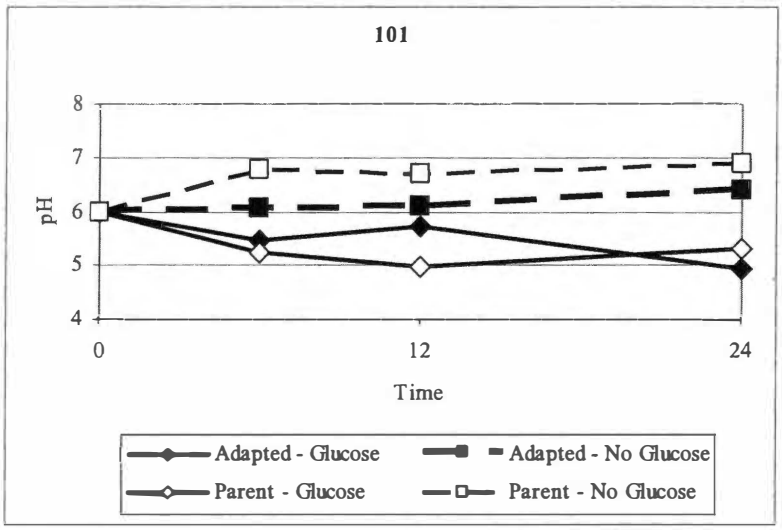


B.

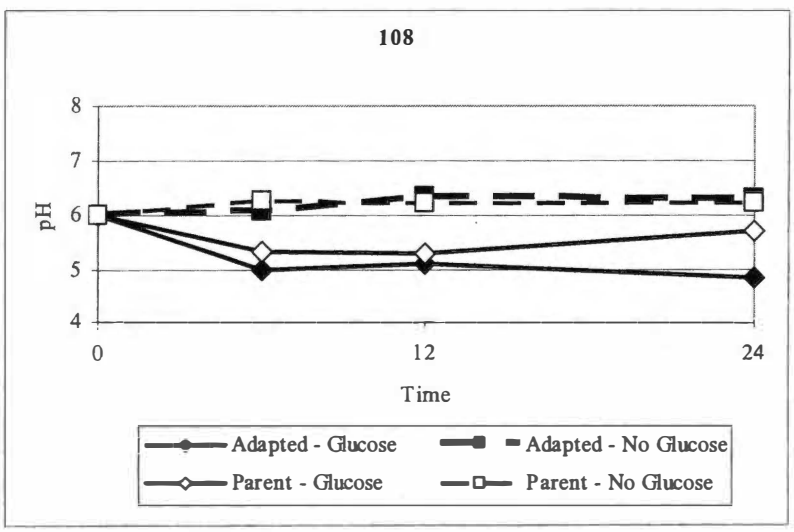


Appendix 18.0: pH measurements of adapted and parent *L. monocytogenes* 101 (A) and 108 (B) strains grown in 0.4% (w/v) sodium benzoate (BS) in TSB with and without glucose in 24 h.

A.

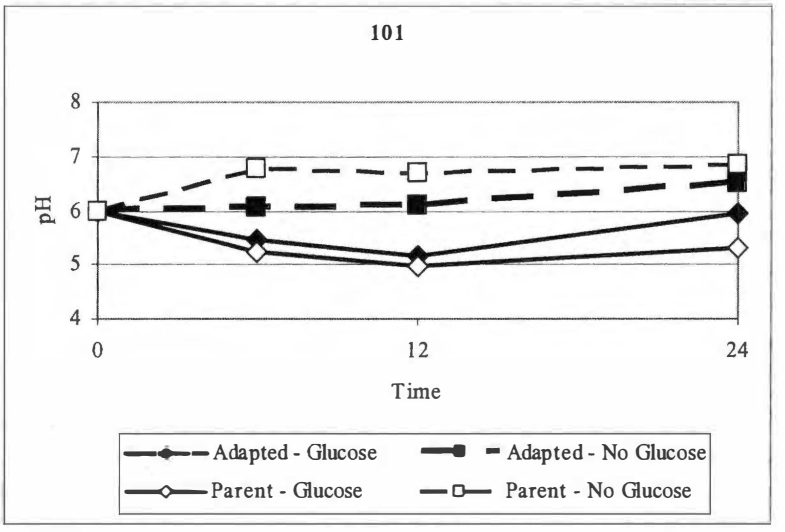


B.

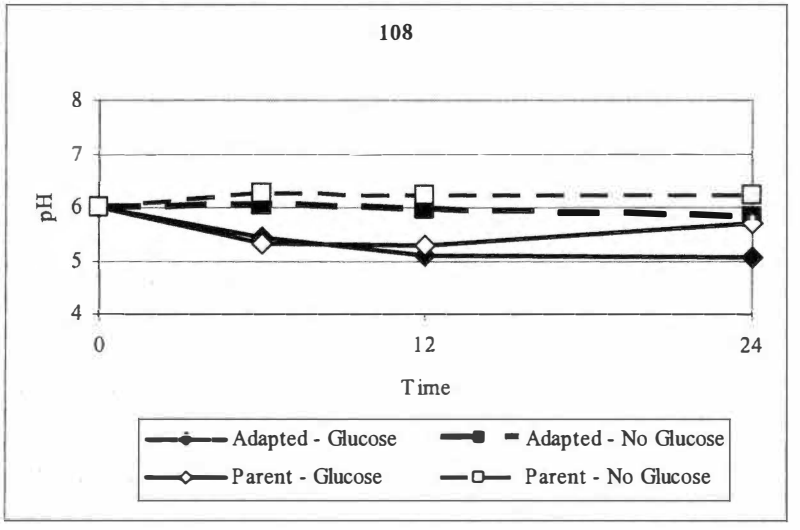


Appendix 19.0: pH measurements of adapted and parent *L. monocytogenes* 101 (A) and 108 (B) strains grown in 4.0% (w/v) sodium lactate (SL) in TSB with and without glucose in 24 h.

A.

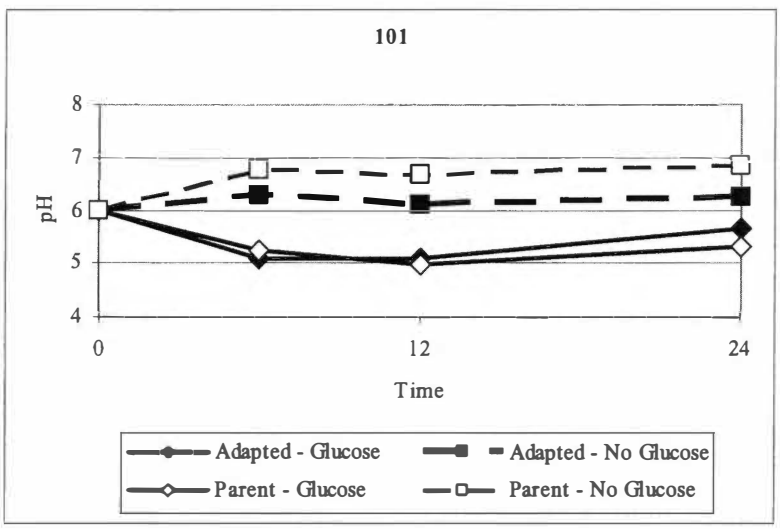


B.

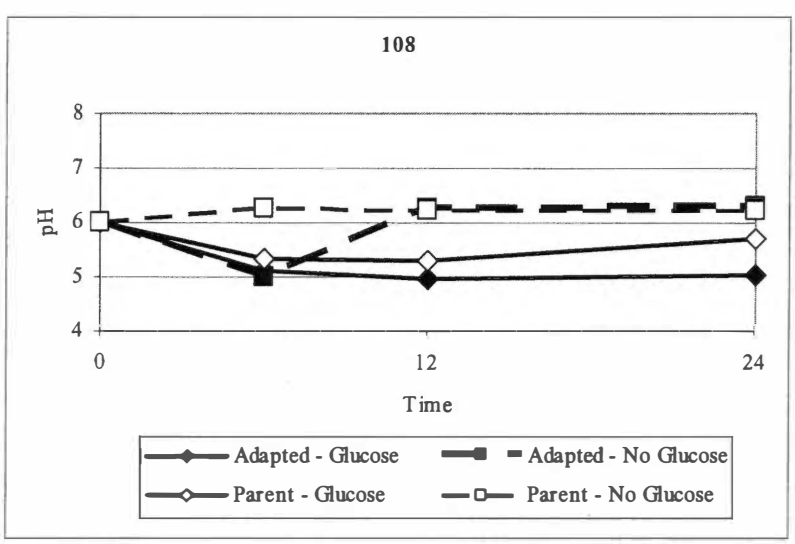


Appendix 20.0: pH measurements of adapted and parent *L. monocytogenes* 101 (A) and 108 (B) strains grown in 0.5% (w/v) sodium diacetate (SD) in TSB with and without glucose in 24 h.

A.

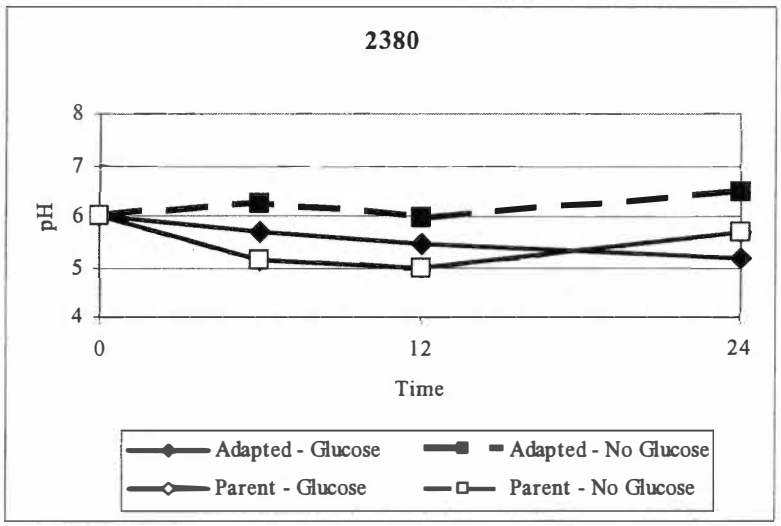


B.

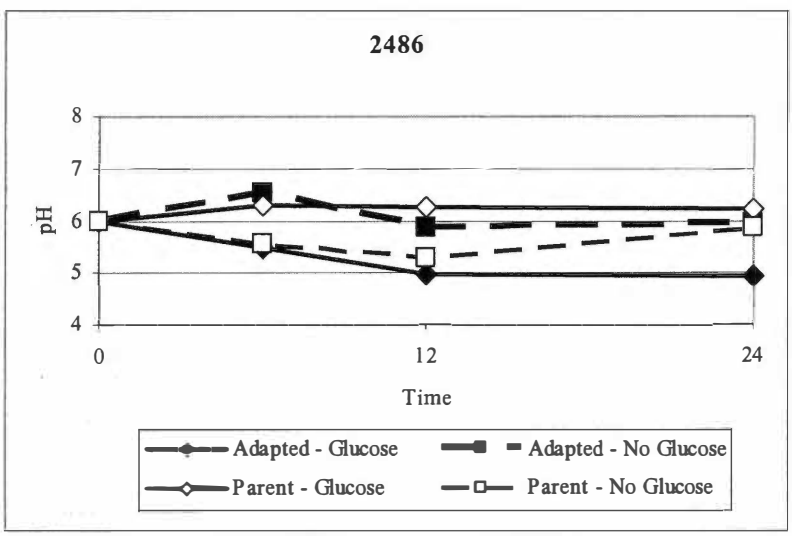


Appendix 21.0: pH measurements of adapted and parent *S.* Typhimurium 2380 (A) and 2486 (B) strains grown in 0.4% (w/v) potassium sorbate (PS) in TSB with and without glucose in 24 h.

A.

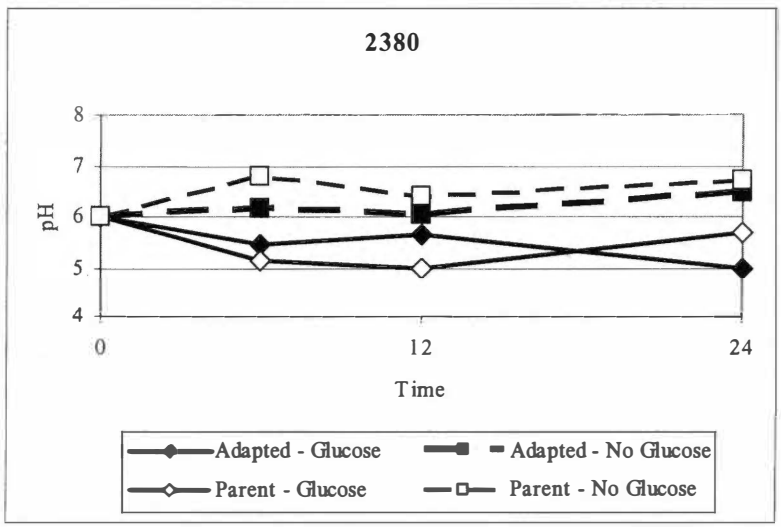


B.

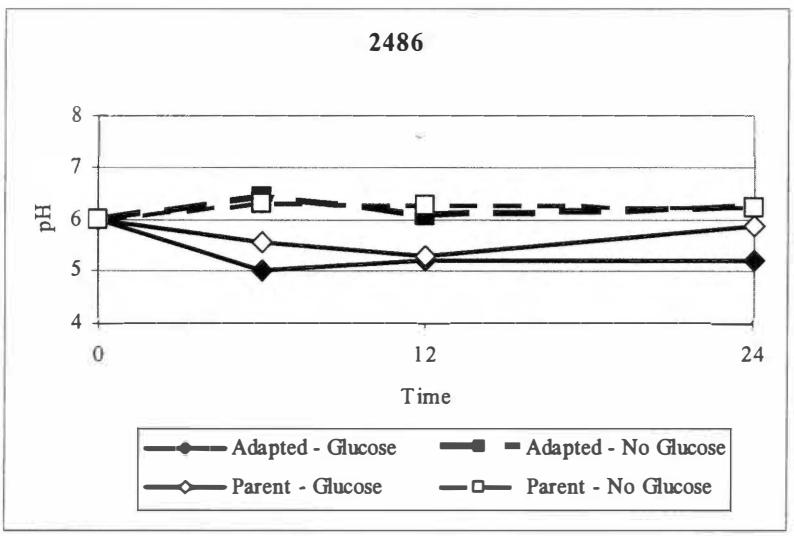


Appendix 22.0: pH measurements of adapted and parent *S.* Typhimurium 2380 (A) and 2486 (B) strains grown in 0.4% (w/v) sodium benzoate (SB) in TSB with and without glucose in 24 h.

A.

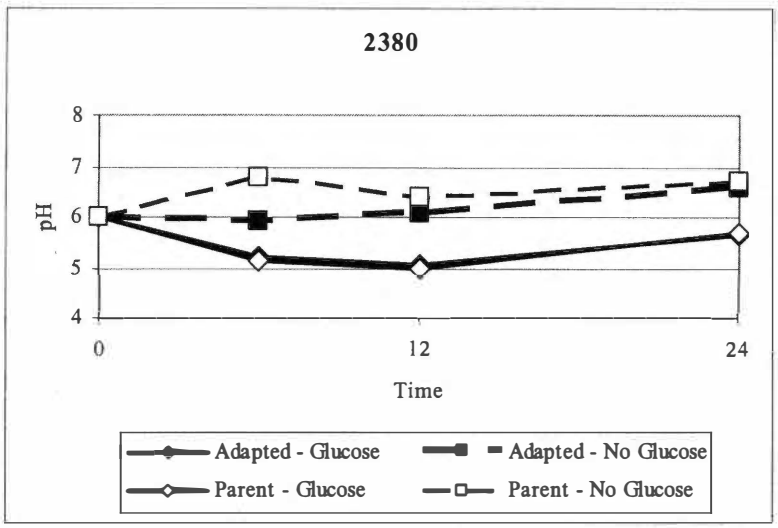


B.

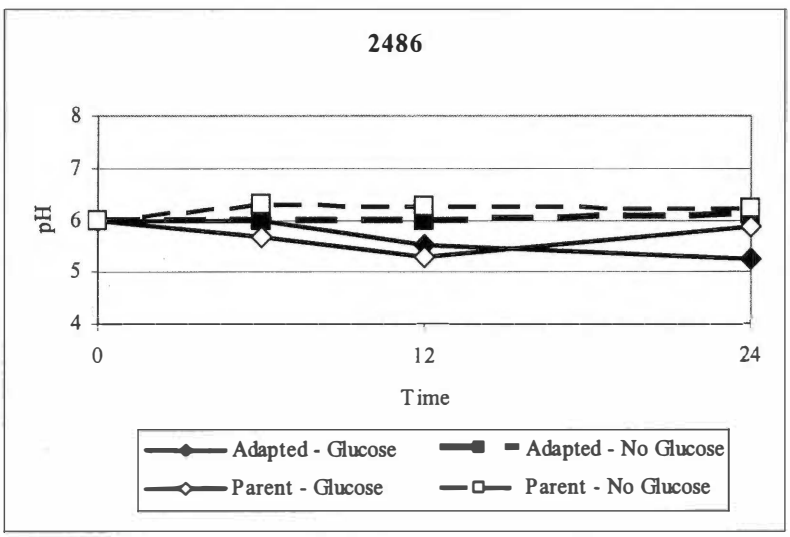


Appendix 23.0: pH measurements of adapted and parent *S.* Typhimurium 2380 (A) and 2486 (B) strains grown in 4.0% (w/v) sodium lactate (SL) in TSB with and without glucose in 24 h.

A.

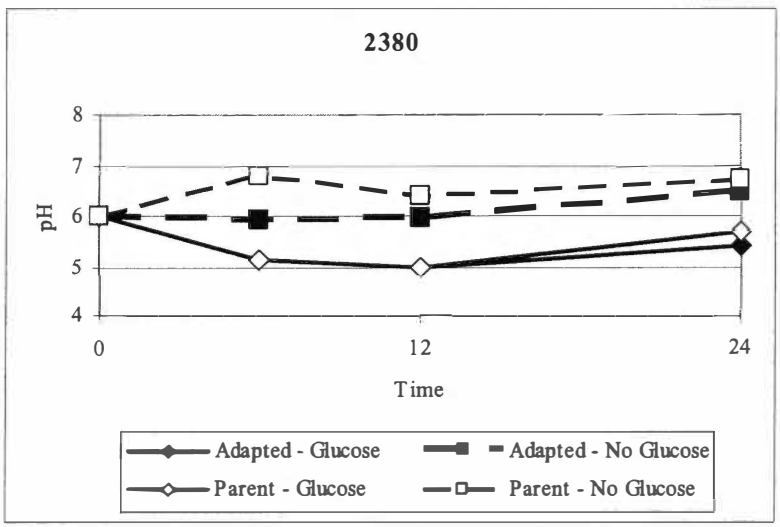


B.

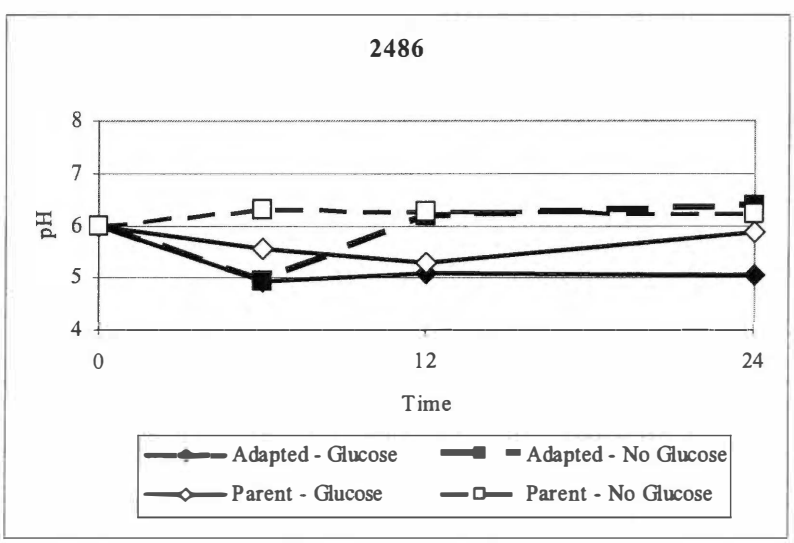


Appendix 24.0: pH measurements of adapted and parent *S.* Typhimurium 2380 (A) and 2486 (B) strains grown in 0.5% (w/v) sodium diacetate (SD) in TSB with and without glucose in 24 h.

A.



B.



PART FOUR: CONCLUDING REMARKS

I. Concluding Remarks

Newly emerging microbiological strains, such as *Listeria monocytogenes*, virulent strains of *E. coli* and multidrug resistant *Salmonella enterica* serovar Typhimurium DT 104 have prompted the need to improve the microbiological status on many processed foods by increasing use of antimicrobial preservative compounds. The Center for Disease Control and Prevention estimates that the yearly cost of foodborne illnesses in the U.S. is 5-6 billion dollars. Resistant pathogens surviving traditional regulatory-approved food antimicrobials and sanitizers represent a threat to the food industry and consumers. Mechanisms of resistance to food antimicrobials are not fully understood but investigations into the potential for such resistance are of extreme importance to the future use of traditional food antimicrobials (Davidson and Harrison, 2003).

Food antimicrobials should not contribute to the development of resistant strains nor alter the environment of the food in such a way that growth of another pathogen is selected (Davidson and Branen, 2005). It is crucial to continue investigations of the potential for resistance development and monitor the proper and adequate use of these antimicrobial compounds in order to preserve the safety of the food supply of the future.

LIST OF REFERENCES

List of References

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Davidson, P.M. and M.A. Harrison. 2003. Microbial Adaptation to Stresses by Food Preservation, p.55-73. In A.E. Yousef and V.K. Juneja (1st ed), Microbial Stress Adaptation and Food Safety. CRC Press, Boca Raton, FL.

Vita

Lilia Milagros Santiago Santiago was born in San Juan, Puerto Rico on March 31, 1970. She was raised in the city of Caguas, Puerto Rico and went to grade school at "Colegio Catolico" and junior and high school at Notre Dame High School in Caguas. She graduated from high school in 1988. From there, she went to Seton Hill College in Greensburg, Pennsylvania and then transferred after her freshman year to Iowa State University in Ames, Iowa. She received a B.S. in Animal Science in 1993, a B.S. in Dairy Science in 1995 and M.S. in Animal Science with a major in Meat Science in 1995. She worked as a Quality Assurance Manager for ConAgra Poultry Company in Aibonito, Puerto Rico from 1996 to 2001. Then moved back to the US and worked as a Ground Turkey Production Supervisor for Cargill Turkey Company in Dayton, Virginia from 2001 to 2002.

Lilia will obtain her doctorate degree in Food Science and Technology, major Food Microbiology at the University of Tennessee, Knoxville, on May 2006. She is a member of the Agricultural Honor Society "Gamma Sigma Delta," National Chancellor List, Institute of Food Technologist, International Association of Food Protection, American Society of Microbiology and National Environmental Health Agency. Currently she works as "Senior Research Scientist" for John Morrell and Company in Cincinnati, Ohio.